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Phylogeography and population genetic study of Siberian roe deer (*Capreolus pygargus*) and phylogeography study of Siberian musk deer (*Moschus moschiferus*)

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Phylogeography and population genetic study of Siberian roe deer (*Capreolus pygargus*) and phylogeography study of Siberian musk deer (*Moschus moschiferus*)

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Abstract

Roe deer, *Capreolus* sp., is one of the most widespread meso-mammals of Palearctic distribution, and includes two species, the European roe deer, *C. capreolus* inhabiting mainly Europe, and the Siberian roe deer, *C. pygargus*, distributed throughout continental Asia. Although there are a number of genetic studies concerning European roe deer, the Siberian roe deer has been studied less about genetic diversity and genetic relationship, and none of these studies use microsatellite markers. In this study, phylogeography,

genetic diversity and population genetic structure of Siberian roe deer was examined using mitochondrial DNA and microsatellite marker.

Genetic diversity and phylogeography of Siberian roe deer was conducted based on combined sequences of mitochondrial cytochrome *b* (1,140bp) and mtDNA control region (963bp) of 219 roe deer from 12 locations (grouped into 7 populations) in Russia, Mongolia and South Korea. Most of Siberian roe deer populations revealed moderate level of haplotype and nucleotide diversity in control region compared to those previously reported for Siberian roe deer and other Cervidae. Especially, roe deer from Jeju Island, South Korea (SKJ) showed the lowest level of genetic diversity and distant from the all other roe deer owing to founder effect and geographic isolation for a long period. Siberian roe deer from Jeju Island (SKJ) had unique and conservation of one mitochondrial lineages, albeit it was not appeared to be distinct phylogenetic clade. Siberian roe deer in the area from Urals to Pacific Ocean was genetically not described subspecies distribution and phylogeograpic pattern in the phylogenetic tree and network. However, Siberian roe deer have four haplogroups, also various haplogroup exist in the east Siberia regions and two haplogroups mainly exist in the west Siberia regions. Trans-Baikal region (RSMG) and Amur region (RPRA) have high diversity, various haplogroups and demographic growth. Therefore, putative ancestral groups were presumably exsited in mountains range of the southern Siberia, and/or Trans-Baikal region (RSMG) and Amur region (RPRA) were geographical

location of secondary colonization.

To examine the level of population genetic structure and the amount of genetic variation of Siberian roe deer, 12 microsatellite loci were analysis from 189 samples throughout Asia. The result showed Moderate levels of genetic diversity (Ar= 2.8−3.7, H_E= 0.52−0.63) were found in all populations except in Jeju Island, South Korea, where the diversity was lowest (Ar= 2.2, H_E= 0.39). Western populations showed relatively low genetic diversity (mean Ar= 2.9, H_E= 0.54) and higher degrees of genetic differentiation (mean pairwise F_{ST}= 0.122) compared with eastern populations. Three genetically distinct groups were existence in Siberian roe deer, which comprise of the Southeastern group (Mainland Korea, Russian Far East, Trans−Baikal region and Northern part of Mongolia), Northwestern group (Western Siberia and Ural in Russia) and Jeju Island population. The results (Barrier, AMOVA, F_{ST} and gene flow) supported genetic differentiation among regions separated primarily by major mountain ridges (Altai, Sayan, stanovoy and Kolyma ridge), suggesting that mountains played a role in the genetic differentiation of Siberian roe deer. Meanwhile, ongoing migration between two groups was presented at the border areas with genetic admixture. Overall, at least three management units of roe deer were suggested in continental Asia, although genetic admixture is evident in border areas between two groups.

Siberian musk deer, *Moschus moschiferus*, is an internationally recognized endangered species. One large reason that musk deer are endangered is the overhunting by human and loss of habitat.

They are one of the most widespread species of the genus *Moschus* in the family Moschidae. In South Korea, Siberian musk deer are locally abundant in the high mountainous and estimated to be lived along the Mt. Taebaek before. However, the distribution of Korean subspecies (*M. m. parvipes*) had a greatly decreased from 1950s to 1999, thus effective conservation is needed. For the successful and accurate conservation, it is important to check the genetic status of population. Genetic analysis can provide genetic diversity and genetic relationship and that can support the carrying adequate restoration and conservation programs.

To investigate the genetic relationship of the Korean subspecies with other subspecies and the extent of genetic diversity, we obtained mitochondrial control region sequence (300bp) from 13 hair and DNA samples from three location and different subspecies (Russian Far East, *M. m. turovi*; Northeastern China, *M. m. moschiferus*; South Korea, *M. m. parvipes*). To obtain a comprehensive genetic relationship between subspecies, published control region sequence (300bp) of 35 individual from NCBI were used to analysis. The results could not discuss whether Korean subspecies belong to a single subspecies or not due to low bootstrap value, small sample size and genetically closest to Russian Far East. But, there was distinction pattern of haplotype composition among subspecies. Network result reveals the Siberian musk deer in Korea originated from Russian Far East, which originated from Siberia (ancestral type). Korean subspecies showed high nucleotide diversity (π= 1.3%) and low haplotype diversity

(Hd= 0.67) compared with similar sample size of Sakhalin Island. This indicate strong bottleneck in a formerly large, stable population. If a decreased population size is maintained, it is obvious that genetic variability will be rapidly destroyed after more generations in the future. Thus we suggested musk deer of Russian Far-East (specifically Primorsky Krai), which are genetically close and originate form of Korean subspecies, as a potential population for restoring. The insights obtained from this study shed light on management of Siberian roe deer in Asia and Siberian musk deer in South Korea and may be applied in conservation of local populations of these two species.

Keywords: Siberian roe deer, Siberian musk deer, Genetic diversity, Phylogeography, Endangered species, Microsatellite, Mitochondrial DNA, Management unit

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General Introduction

Artiodactyls are exceptionally diverse and globally distributed every continent expect Antarctica, and they first appeared during the early Eocene (about 55million years ago). Artiodactyls consists 10 families, approximately 220 species. Artiodactyls called the even-toed ungulates have third and fourth toes and other toes are reduced or lost.

In this study, we investigated two Artiodactyl species in Cervidae (Siberian roe deer, *Capreolus pygargus*) and Moschidae (Siberian musk deer, *Moschus moschiferus*). These two species are widely distributed in similar areas from Siberia, Russian Far East, Northeast China, and Mongol. Although their distribution range is similar to each other, the two species are different in ecological feature such as behavior, breeding, and habitat selection, and so on. Besides, Siberian musk deer is an endangered species, and a species of which the conservation is urgent. However, in comparison with musk deer, in case of Siberian roe deer, their population is relatively abundant. They are considered to be food resources for endangered carnivore. Hence, it is necessary to proper management of two species, and very important to know genetic status as well as current situation for effective management. Therefore, this study is intended to identify the genetic structure, phylogeography, and genetic diversity of Siberian roe deer, and the phylogeography and genetic diversity of Siberian musk deer.

Siberian roe deer (*Capreolus pygargus*)

The Siberian roe deer, *Capreolus pygargus*, is a moderate size deer, with a long neck and large ears. Siberian roe deer belongs to the family Cervidae with 40 species of deer and it was once considered by the same species with the European roe deer (*Capreolus capreolus*). Morphologically, Siberian roe deer has larger antlers with more branches and large body size than those of European roe deer. Genetically, Siberian roe deer has 1−14 more subsidiaries B−chromosome than European roe deer.

Although intraspecies taxonomy (subspecies) is still controversial, it is widely accepted that the Siberian roe deer comprises of at least two subspecies, *C. p. tianschanicus* (or *C. c. bedfordi* Thomas, 1908) (Tianshan mountain, Mongolia, Russian Far East and Korea) and *C. pygargus pygargus* (from Volga river to Lake Baikal and Northeastern Russia). Siberian roe deer in Central China and Tibet are sometimes described as different subspecies, *C. p. melanotis* Miller, 1911 (Heptner *et al.*, 1988; Danilkin, 1999; Sheremetyeva *et al.*, 2010). Also Jeju Island, South Korea was suggested in fourth subspecies, *C. p. ochracea* (Koh and Randi, 2001).

The Siberian roe deer is known to have a very wide distribution and it is distributed in continental Asia and parts of Eastern Europe (Danilkin, 1995), from the Don River to the coastlines of the East Sea, and the Yellow Sea, including the Korean Peninsula (Danilkin, 1995). Fossil records show that Siberian roe deer territory was

once reached to the northern Caucasus (Korotkevich and Danilkin, 1992). However, population size drastically diminished supposedly because of overhunting during the 19th and 20th centuries (Danilkin, 1995). Regardless, the original historic distribution has almost completely recovered.

Now, Siberian roe deer is classified as Least Concern (LC) by the IUCN and legal hunting with hunting licenses was formally permitted in Russia and Korea (Jeju Island) seasonally. Commercial hunting is allowed in some of protected areas (special purpose reserves) in Russia and the allocation of animal is based on periodic estimates of the population size (Danilkin *et al.*, 2000, Korytin *et al.*, 2002). In the Jeju Island, South Korea, licensed hunters are allowed to the maximum of three individuals per hunting season. In another aspect of the Siberian roe deer, they play an important role in the ecosystem, providing a prey for large carnivores. Siberian roe deer is one of the main prey of Amur leopard (*Panthera pardus orientalis*), which is one of the most endangered subspecies, in the border area among Russia, China and North Korea (Pikunov and Korkishko, 1990; Heptner *et al.*, 1992; Miquelle *et al.*, 1999; Peterson and Ciucci, 2003; Molinari-Jobin *et al.*, 2007; Hebblewhite *et al.*, 2011). Siberian roe deer also serve as an important prey species for other carnivores like Amur tigers, gray wolves, lynxes, dholes, bears, as well as foxes, martens, eagles and wild boars (Miquelle *et al.*, 1999; Geist 1998).

Thus, proper management of Siberian roe deer populations in northern Asian continent will need to be conservation status and

also benefits for many other species. However, the Siberian roe deer is relatively less studied for genetic status and most of the genetic studies of the species have been obtained from phylogenetic inferences using mitochondrial DNA based on the relatively small samples. In this study, we investigate the genetic diversity, phylogeographic pattern and genetic structure of the Siberian roe deer based on genetic marker (microsatellite loci and mitochondrial DNA).

Siberian musk deer (*Moschus moschiferus*)

Siberian musk deer, *Moschus moschiferus*, is an internationally recognized endangered species and classified as Vulnerable (VU) by the IUCN and CITES Appendix II (Nyambayar *et al.*, 2015). One important reason that musk deer are endangered is the overhunting by human and loss of habitat (Wemmer, 1998; Homes, 2004).

The Siberian musk deer (*Moschus moschiferus*) is one of the most widespread species of the genus *Moschus* in the family Moschidae, and forest animal inhabited in mixed coniferous of mountainous regions. It is distributed widely in the Russian Federation (Siberia and the Far East), eastern Kazakhstan, northeastern and northwestern China, Mongolia and Korea (Tsendjav, 2002; Baskin and Danell, 2003; Nyambayar *et al.*, 2015).

The subspecies classification of Siberian musk deer is

controversial and studies for species are in the initial stage. First, three subspecies based on characteristics of external and skull morphology was suggested (Groves *et al.*, 1995; Groves and Grubb, 2011): *M. m. moschiferus* (Siberia, Mongolia, Northwest Heilongjiang), *M. m. parvipes* (Russian Far East, Korea, South Heilongjing) and *M. m. sachalinensis* (Sakhalin). While, two more subspecies were suggested with the color features, region difference and pelage by Sokolov and Prikhod'ko (1997, 1998): *M. m. moschiferus* (Siberia and mongolia), *M. m. turovi* (Russian Far East), *M. m. arcticus* (Verkhoyansk Ridge), *M. m. parvipes* (Korea) and *M. m. sachalinensis* (Sakhalin).

In South Korea, Siberian musk deer are estimated to have lived along the Mt. Taebaek before and locally abundant in the high mountainous regions. However, the distribution of Korean subspecies (*M. m. parvipes*) had a greatly decreased from 1950s to 1999 for the same reasons (Lee and Rhim, 2002). Up to now, South Korea designated them as the natural monument and class Ⅰ endnagered species (Won, 1992).

Therefore, it is important to check the status of population as well as carry out an ecological study of habitat for conservation of endangered species (Kim *et al.*, 2011). Genetic variation of population or species is also considered important for restoring threatened animal and conservation genetics (Avise, 2004). Genetic information can support the carrying adequate restoration programs (Lee *et al.*, 2008). However, there have been few studies on Korean musk deer habitats (Kim *et al.*, 2007; Park *et al.*, 2008) and

no study has been released on molecular marker based genetic diversity and phylogenetic relationship for musk deer in korea.

Overall, phylogeography studies of the Siberian musk deer can provide fundamental information to better understand the present genetic status and genetic relationship of Korean subspecies (*M. m. parvipes*). This study investigates the level of genetic diversity and the genetic relationship of the Korean population with other subspecies. The results from this study can be practical in the future conservation, re-introduction and management of Siberian musk deer in South Korea.

CHAPTER Ⅰ.

Genetic diversity and Phylogeography of Siberian roe deer (*Capreolus pygargus*) based on mitochondrial DNA

Introduction

The roe deer (*Capreolus*, Gray 1821) is one of the most widespread artiodactyl genera in nature. It includes two species: the European roe deer (*C. capreolus*) and the Siberian roe deer (*C. pygargus*). The Siberian roe deer is known to have a very wide distribution in the Palaearctic. It is widely distributed in continental Asia and parts of Eastern Europe (Danilkin, 1995), from the Khoper and Don River bend to the Ural Mountains and across southern Siberia. It is found through northern Mongolia and east to the coastlines of the East Sea, and the Yellow Sea, including the Korean Peninsula (Danilkin, 1995). Its geographic range branches out towards the south at the West Siberian Plain down to Lake Balkhash, and from there expanding back to the east well into Kazakhstan without reaching the Aral Sea. Also, it inhabits from Manchuria into northern and central China, to the western half of the left margin of the Yang Tze River, into the eastern Tibetan region (Sokolov *et al.*, 1982; Danilkin, 1999). Records from further south as far as northeastern Myanmar require confirmation. It also occurs on Jeju Island in South

Korea.

Although intraspecies taxonomy of Siberian roe deer is questionable, most authors agree that *C. pygargus* consists of at least two subspecies with number of B−chromosome (Groves and Grubb, 2011): *C. p. pygargus*, distributed from the Volga River to lake Baikal and *C. p. tianschanicus*, found in Tien Shan, Mongolia, Trans baikalia, Far East, and China (Danilkin, 1999; Sheremetyeva and Sheremetyev, 2008). Roe deer in Central China and Tibet are sometimes described as separate subspecies, *C. p. melanotis* (Danilkin, 1999; Sheremetyeva *et al*., 2010). Also fourth subspecies has been suggested in Jeju Island, South Korea, as *C. p. ochracea* (Koh and Randi, 2001).

Data on the genetics of Siberian roe deer are scarce in compare with European roe deer. Randi *et al*. (1998) presented the outcome that Siberian roe deer can be divided into two major clusters, i.e. the eastern cluster (Amur region, Russian Far East) and the western cluster of (Kurgan region, Western Siberia, Russia). Studies on the taxonomic status of the Siberian roe deer from Jeju, South Korea and genetic structure of the Siberian roe deer from Northern Eurasia have been previously presented using molecular genetics tools (Tokarskaia *et al*., 2000; Koh and Randi, 2001). Petrosian *et al*. (2002) using RAPD marker confirmed previous results about the diversification of eastern and western groups, correlated to subspecies *C. p. pygargus* and *C. p. tianschanicus* respectively. At the same time Xiao *et al*. (2007) made an argument that the roe deer found in northeastern China belongs to another

subspecies *C. p. manchuricus,* based on morphological difference from other subspecies of Siberian roe deer. While Sheremetyeva *et al*. (2010) presented complex phylogenetic structure of the roe deer populations in Russian Far East using short fragment of control region and challenged "the generally accepted views on the interspecies variability of Siberian roe deer".

More recently, Zvychainaya *et al*. (2011) showed three haplogroups, based on the combined alignment of control region and cytochrome *b* for 79 Siberian roe deer sampled from 23 regions of Asia including Russia and Kazakhstan, that individuals from Russia East, northeast and Transbaikalia formed a single haplogroup, whereas the specimens from Urals, Western and Central Siberia were shared by two distinct haplogroups (both regions were presented in each haplogroup). In addition, Lorenzini *et al.* (2014) suggested three haplogroups for Siberian roe deer are distributed throughout the entire range of this species distribution across Western Russia, Kyrgyzstan, North-eastern China, Central-eastern China and Eastern Russia, but no geographical structuring of the species lineages was found.

Most of the above mentioned studies are based on the relatively small samples (but see Xiao *et al*., 2007) and this could be one of reasons being uncertainty in the phylogeographical patterns (reported particularly by Zvychainaya *et al*., 2011). Previously published data suggest the existence of at least two or three phylogroups, however phylogenetic relationships between these groups remain unclear, particularly in central Siberia, which is

supposed to be the area where the geographical ranges of two subspecies (*C. p. pygargus* and *C. p. tyanschanicus*) overlap (Sheremetyeva, 2010).

With peripheral populations the picture becomes especially complex. For example, Zvychainaya *et al.* (2011) reported that roe deer from Urals and Trans-Urals region (Sverdlovsk and Kurgan regions, close to the western periphery of the species' geographical range) were presented by two haplogroups, each of these haplogroups occupied distal position of the phylogenetic tree. Likewise, recent data on the genetic features of roe deer from Yakutia (northern periphery of the species' geographical range) put them into the Far Eastern clade (Zvychainaya *et al.*, 2011). Thus the phylogeographical structure of the Siberian roe deer is still very ambiguous and many authors emphasize the necessity of extensive studies of the species in a number of regions.

In this study we report the data on the genetic diversity and phylogeographical structure of the Siberian roe deer based on the sufficient number of samples (not less than 20 from most regions). We focus on the genetics of roe deer in Korea (the Korean peninsula and Jeju Island), because previously only few specimen was involved in the analysis. Special interests were given on genetic features of the peripheral (Ural and Yakutia) and isolated (Jeju Island) populations of the roe deer.

Materials and Methods

Sample collection and DNA extraction

A total of 219 individuals of *C. pygargus* were obtained from 12 locations (Table 1 and Appendix S1) in Russia, Mongolia, and South Korea. These locations were grouped into seven populations according to the geographic proximity: South Korea, Jeju (SKJ), mainland of South Korea (SKM), Russia, Primorsky Krai and Amur region (RPRA), Russia, Yakutia (RYA), Russia, Trans-Baikal region, Sokhondinsky nature reserve and Northern Mongolia (RSMG), Russia, Altay and Novosibirsk (RARN), Russia, Ural, Kurgan and Orenburg (RUKO). All samples were frozen at −70℃ deep freezer of Conservation Genome Resource Bank for Korean wildlife (CGRB) until DNA extraction. Genomic DNA was extracted from tissue, blood and skin using the QIAamp tissue kit (Qiagen, Germany).

Table 1. Sampling information of each location per population and haplotype distribution. Mitochondrial DNA control region and cytochrome *b* were combined for analysis. Bold and underline types are shared haplotype among region

Region	Location (Abbreviation)	N	Haplotype
SKJ	South Korea, Jeju (SKJ)	37	Hap80(15), Hap81(1), Hap82(6), Hap83(2), Hap84(3), Hap85(4), Hap86(1) Hap87(4), Hap88(1)
SKM	South Korea, mainland (SKM)	30	Hap17(1), Hap19(3), Hap20(1), Hap33(1), Hap34(1), Hap45(1), Hap49(3), Hap50(1), Hap51(4), Hap52(1), Hap53(3), Hap54(1), Hap55(1), Hap56(1), Hap58(3), Hap59(1), Hap60(1), Hap95(1), Hap108(1)
RPRA	Russia, Primorsky Krai (RPR)	41	Hap1(1), Hap5(1), Hap6(1), Hap7(1), Hap18(1), Hap21(1), Hap24(1), Hap25(1), Hap26(1), Hap28(1), Hap36(1), Hap37(1), Hap38(1), Hap39(1), Hap42(3), Hap43(1), Hap46(3), Hap48(1), Hap57(1), Hap61(1), Hap62(2), Hap63(1), Hap65(2), Hap66(1), Hap67(1), Hap68(1), Hap69(1), Hap70(1), Hap71(1), Hap90(1), Hap92(1), Hap93(1), Hap94(1), Hap96(1), Hap97(1)
RPRA	Russia, Amur region (RAM)	10	Hap3(1), Hap9(1) , Hap22(1), Hap31(1), Hap44(1), Hap47(2), Hap91(1), Hap104(1), Hap105(1)
RYA	Russia, Yakutia	24	Hap10(7), Hap11(1), Hap29(3), Hap30(1), Hap89(5), Hap101(5), Hap102(1), Hap107(1)
RSMG	Russia, Sokhondinsky (RSO)	10	Hap12(1), Hap13(1), Hap23(1), Hap40(1), Hap41(1), Hap64(1), Hap74(1), Hap98(1), Hap103(1), Hap106(1), Hap109(1), Hap110(1)
RSMG	Mongolia, Northern part (MGN)	12	Hap2(1), Hap4(1), Hap8(1), Hap14(1), Hap15(1), Hap16(1), Hap27(1), Hap32(1), Hap35(1), Hap72(1)
RARN	Russia, Altay (RAL)	3	Hap75(1), Hap98(1), Hap112(1)
RARN	Russia, Novosibirsk (RNO)	6	Hap75(4), Hap79(1), Hap111(1)
RUKO	Russia, Ural (RUR)	23	Hap73(2), Hap75(5), Hap76(1), Hap79(3), Hap99(4), Hap100(8)
RUKO	Russia, Kurgan (RKU)	20	Hap73(6), Hap75(1), Hap77(1), Hap78(1), Hap79(2), Hap99(8), Hap100(1)
RUKO	Russia, Orenburg (ROR)	3	Hap75(1), Hap76(1), Hap77(1),
C.c	Ukraine, Crimea	3	Hap113(2), Hap114(1)

N, sample size; C.c, *Capreolus capleolus* (out-group)

PCR amplification and DNA sequencing

The cytochrome *b* gene (1,140bp) was amplified by polymerase chain reaction (PCR) using universal primer L14724 (5' – GAT ATG AAA AAC CAT CGT TG – 3') and H15915 (5' – AAC TGC AGT CAT CTC CGG TTT ACA AGA C – 3') (Kocher *et al.* 1989).The PCR reaction conditions were: 94℃ for 4 min; 35cycles of 94℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 1 min; and finally 72℃ for 5 min. The 923 bp fragment of mtDNA control region was amplified using primers L15775 (5' – ACA TGA ATT GGA GGA CAA CCA GT – 3') (Irwin *et al.*, 1991) and H651 (5' – AAG GCT AGG ACC AAA CCT – 3') (Kocher *et al.*, 1989). The PCR reaction conditions were: 94℃ for 5 min; 35cycles of 94℃ for 45 s, 55℃ for 45 s, and 72℃ for 1 min 30 s; and finally 72℃ for 5 min. The amplification was carried out in 20 ㎕ reaction volume containing 10 – 100 ng template DNA, 100 μM each dNTPs, 10 pmole each primer, 1.5 mM $MgCl_2$, 1 unit i-star Taq™ DNA polymerase (iNtRON Biotechnology Inc, Korea), and 1 x PCR buffer. The PCR products were purified with Zymoclean™ Gel DNA Recovery Kit (ZYMO RESEARCH, USA). Purified PCR products were sequenced using ABI Prism™ 377 automated sequencer (Applied Biosystems Inc, USA). The sequencing primers for both mtDNA regions were the same as those used for the amplification however, in case of control region, primers used for the sequencing include supplementary inner primer; L – 362(5' – AAT CAC CAT GCC GCG TGA AAC C – 3') (Douzery and Randi, 1997).

Data analysis

The sequences determined in this study were identified as *Capreolus* species through BLAST searches (Altschul *et al.*, 1997). Sequences were aligned with ClustalX version 1.83 (Thompson *et al.*, 1997). All downstream analyses were conducted with concatenated sequences of two mtDNA regions (2,063bp).

Haplotype diversity (Hd), and nucleotide diversity (π) for each of geographical samples were estimated with DNASP version 5.1 (Librado and Rozas, 2009). The ARLEQUIN 3.1 (Excoffier *et al.*, 2005) was used to calculate mismatch distribution and pairwise F_{ST} to compare genetic differentiation among geographical regions. Mismatch distributions were analyzed using the sudden expansion model (Rogers and Harpending, 1992), and goodness-of-fit tests of the observed to the estimated mismatch distributions were computed. The possible occurrence of historical demographic expansions was also examined using Tajima's D (Tajima, 1989) and Fu's *F*s (Fu, 1997) neutrality tests using the ARLEQUIN. Fu's *F*s is sensitive to demographic expansion, which usually leads to large negative values (Fu, 1997).

Phylogenetic relationships between geographical samples were estimated using the median-joining network procedure using the program Network version 4.6.1.2 (http://www.fluxus-engineering.com/). Network analysis effectively portrays the relationships among sequences, and allows inferring haplotype genealogies at the population level because they explicitly allow for extant ancestral sequences and alternative connections (Bandelt *et al.*, 1999).

Phylogenetic trees to investigate evolutionary relationships were constructed using four methods: Neighbor-joining (NJ: Saitou and Nei, 1987) using Kimura's two parameter distances (Kimura 1980), Maximum parsimony (MP), Maximum-likelihood (ML) and Bayesian inference (BI). We used the combined sequences (2,071bp) as well as cytochrome *b* (1,140bp) and control region (923bp) for phylogenetic trees analysis without tandem repeats. European roe deer (*Capreolus Capreolus*) was used as out-group for phylogenetic tree construction. The NJ, MP and ML trees were performed using MEGA 5.05 (Tamura *et al.*, 2011). The MP tree was obtained using the Close-Neighbor-Interchange (CNI) with random sequence addition and with 10,000 bootstrap replicates. This algorithm has prohibitively long computation times for searching first producing a temporary tree.

The most appropriate models of sequence evolution for ML and Bayesian trees were selected with JMODELTEST 2.1.4 (Posada, 2008). The best-fit model for ML tree was the General Time Reversible model (GTR) with Gamma distributed (+G) and proportion of Invariant sites (+I). The consensus ML trees were found by Nearest-Neighbor-Interchange (NNI) heuristic searches of 1,000 bootstrap replicates.

BI and Bayesian posterior probabilities (BPPs) were estimated using MRBAYES v 3.2.2 (John and Fredrik, 2001). The Hasegawa-Kishino-Yano model (HKY) +G +I was selected as best-fit model for BI tree. Two Markov chains were conducted for 2,000,000 generations and the tree was sampled every 100 generations with

burn-in of first 500 data points. The nodes with bootstrap value (BS) higher than 50% were regarded as sufficiently resolved (Hillis and Bull, 1993). Nodes with BPP higher than 95% were considered statistically significant (Leaché and Reeder, 2002).

The divergence time (T) between mtDNA lineages was estimated among clades of Siberian roe deer shown in the Bayesian tree. The time of divergence was calculated using the equation, $T = K/(2r)$, given by Li (1997), where sequence divergence (K, substitutions/site) was derived from the mean value of P-distance between groups with mean distance using Mega 5.2 (Tamura *et al.*, 2011), and r is the average mutation rate of the mtDNA (0.04-0.08) proposed by Randi *et al.* (1998)

Results

Genetic variability of Siberian roe deer

The combined alignment of mitochondrial control region (923bp) and cytochrome *b* sequences (1,140bp) presented 112 haplotypes, 181 polymorphic site and 187 mutations (excluding sites with gaps and missing data). Haplotype distribution of each population and estimates of genetic diversity in the studied geographical populations are presented in Table1 and Table 2.

Most of Central and Eastern Siberian roe deer (RPRA, RSMG and SKM) did not share haplotypes from one another, except for Russia, Sokhondinsky (RSO), in which one haplotype (Hap98) shared with Russia, Altay (RAL). On the other hand, Western Siberia (RARN and RUKO) populations shared several haplotypes with each other. Yakutia (RYA) and Jeju Island, Korea (SKJ) shared common haplotypes within population, but did no overlap with other populations (Table 1).

In both combined and control region sequence the highest levels of genetic diversity, apart from combined nucleotide diversity, were observed in the Trans-Baikal region (RSMG). Russian Far East (RPRA), Yakutia (RYA), western (RARN) and Ural (RUKO) populations were showed relatively high or moderate level of haplotype diversity (Hd= 0.722-0.993 and 0.722-0.984) and nucleotide diversity (π= 0.745-0.974% and 0.935-1.229%). Mainland Korea (SKM) was characterized with relatively low

nucleotide diversity (π= 0.491% and 0.699%) but relatively high haplotype diversity (Hd= 0.959 and 0.915) compared with other populations. Jeju Island, Korea (SKJ) showed the lowest level of genetic diversity.

Table 2. Estimates of genetic diversity of regional Siberian roe deer. Genetic diversity of control region was also presented for comparing with previous studies. See Table 1 for regional abbreviation.

Region	N	Combined sequence CR + Cyt−*b*			Control region	
		H	Hd	π (%)	Hd	π (%)
SKJ	37	9	0.796	0.082	0.251	0.028
SKM	30	19	0.959	0.491	0.915	0.699
RPRA	51	44	0.993	0.769	0.984	0.935
RYA	24	8	0.841	0.974	0.786	1.229
RSMG	22	22	1	0.899	0.991	1.261
RARN	9	5	0.722	0.745	0.722	0.960
RUKO	46	8	0.843	0.884	0.827	0.988
Total	219	112	0.982	0.968	0.961	1.200

N, sample size; H, Number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity

Phylogeography of Siberian roe deer

Phylogenetic trees using NJ, MP, ML and Bayesian approaches generated similar patterns of the major branches, and therefore Bayesian tree was representatively presented in this study. Bayesian phylogenetic tree of combined sequence (cytochrome *b* and control region) and cytochrome *b* were showed same pattern of the major branches (Figure 1, 2 and 3). However, bayesian phylogenetic tree of control region (Figure 4) was presented difference of haplotype composition in the haplogroup B and C. One haplotype (Hap 52) from Trans-Baikal region (RSMG) and three haplotypes (Hap 53, 53, 55) from Russian Far East (RPRA) were belonging to haplogroup C, not haplogroup B. Although phylogenetic tree of control region was not identical with other phylogenetic trees, phylogenetic tree with combained sequence (control region and cytochrome *b*) was representatively presented, due to the combined sequence of control region and mitochondrial coding region (12S rRNA, 16S rRNA, ND4, ND5, ND6, and cytb) was optimizes the information necessary for phylogenetic analyses (Non *et al.*, 2007). Bayesian tree revealed four major haplogroups with very high posterior probability values (Figure 1). Geographical analysis of the distribution of these haplogroups (Figure 2 and Table 3) indicated that none of them is limited to only one geographical location. Population of roe deer from the Jeju Island consisted only of haplotypes belonging to the haplogroup B. Haplogroup A was found only in the eastern part of *C. pygargus* geographical range. Haplotypes belonging to haplogroup B were

found throughout all populations, excluding mainland South Korea (SKM). Interestingly, the highest frequencies of these haplotypes were found in two populations on the western and eastern periphery of the species geographical range in Urals (RUKO) and on the Jeju Island (SKJ). Haplogroup C was found with a relatively high frequency in all samples, except SKJ. Finally, haplogroup D was found mainly in the eastern part of the species range.

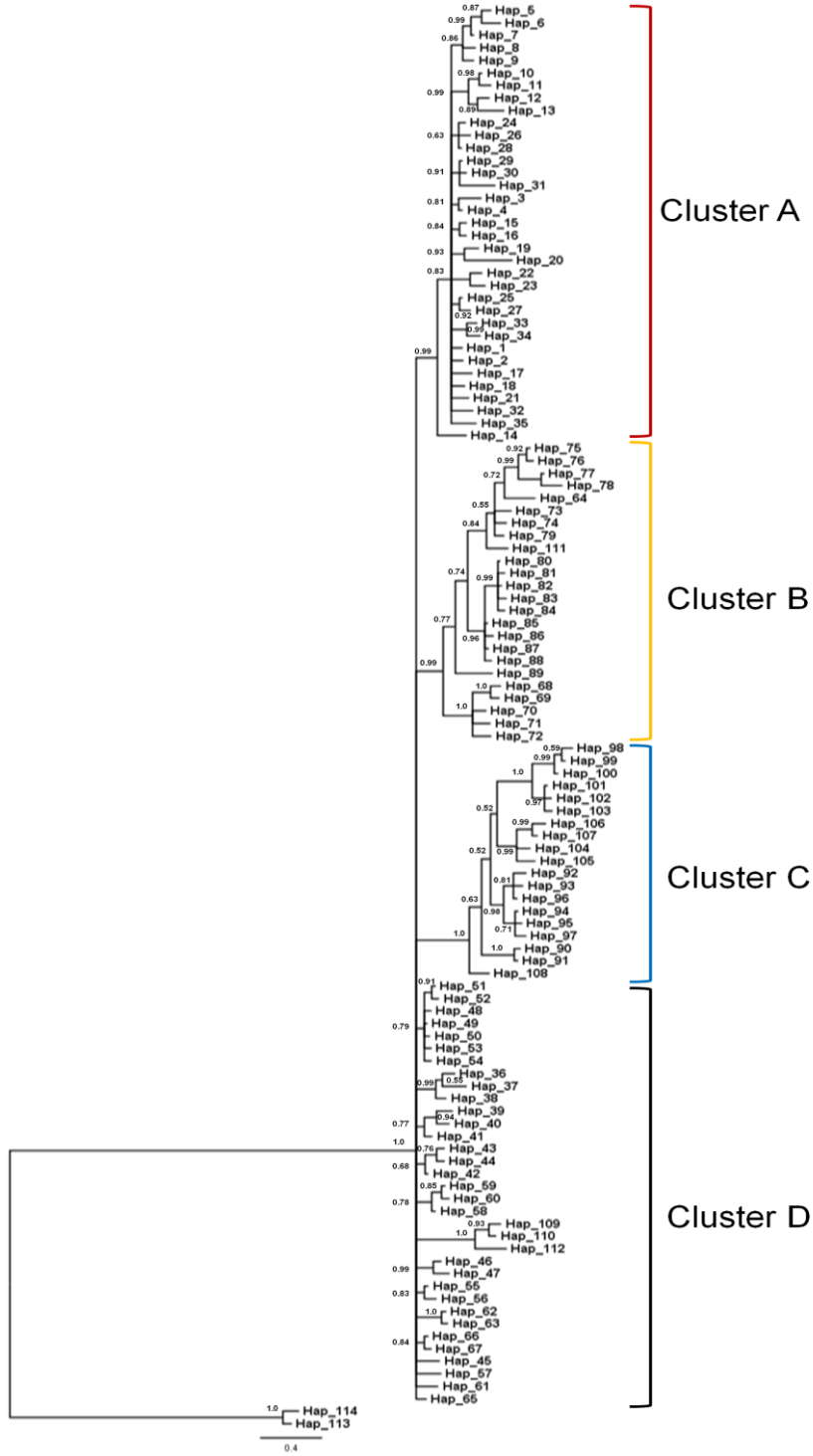


Figure 1. Bayesian (MCMC) haplotype tree of Siberian roe deer based on mtDNA control region (923bp) and cytochrome *b* (1,140bp). Bayesian posterior probability is shown for branches with over 50% support.

Table 3. Distribution of haplotypes in each region among cluster revealed by Bayesian tree analysis. See Table 1 for regional abbreviation.

Cluster	H	population
A	35	SKM(5), RPRA(14), RYA(4), RSMG(12)
B	24	SKJ(9), RPRA(4), RYA(1), RSMG(3), RARN(3), RUKO(6)
C	19	SKM(2), RPRA(9), RYA(3), RSMG(3), RARN(1), RUKO(2)
D	34	SKM(12), RPRA(17), RSMG(4), RARN(1)

H, number of haplotype

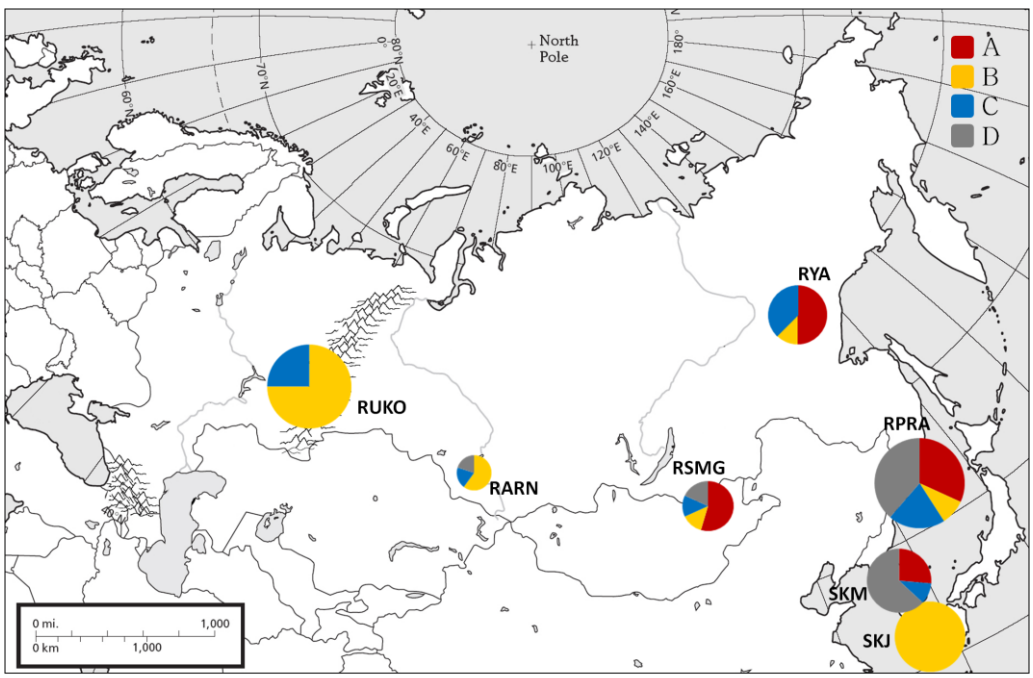


Figure 2. Geographical distribution of the haplogroups revealed by Bayesian analysis. The proportion of color in each circle indicates cluster (A, B, C, D) of phylogenetic tree in Figure 1. The proportion of circle size is number of samples in each region. See Table 1 for regional abbreviation.

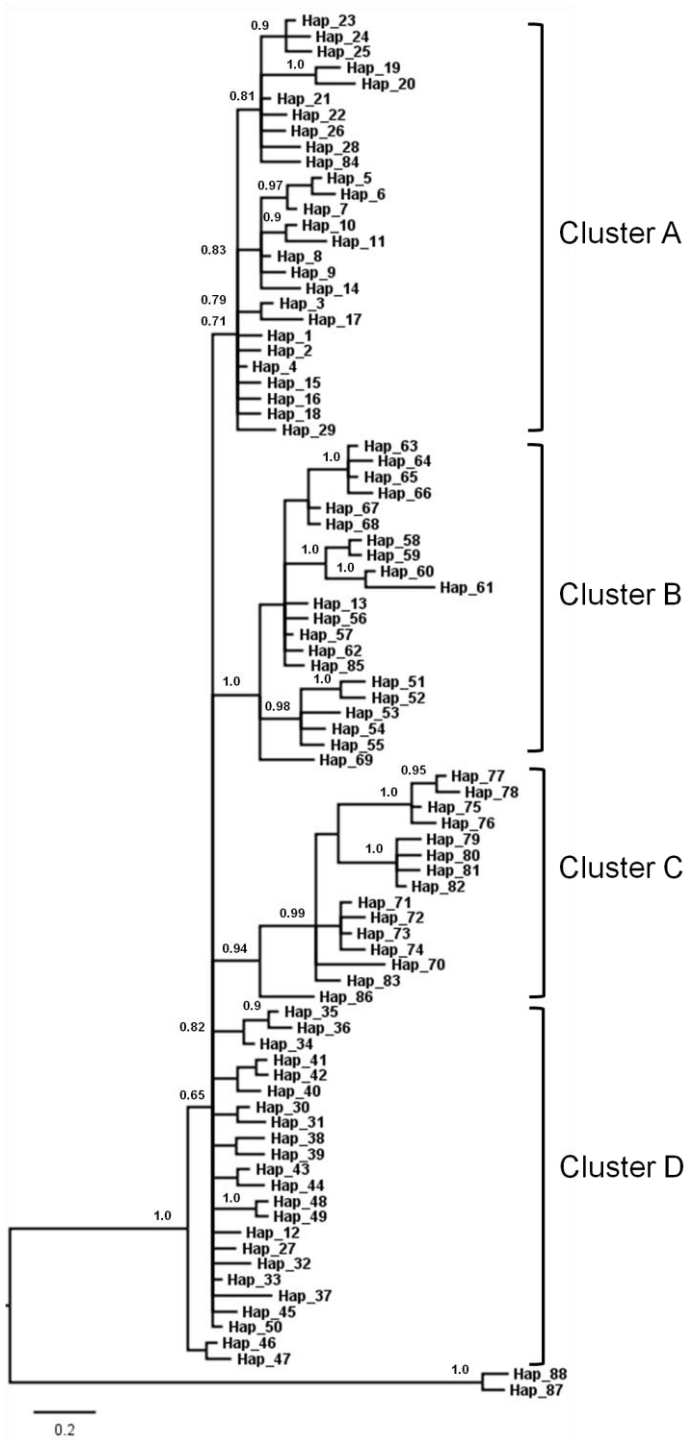


Figure 3. Bayesian (MCMC) haplotype tree of Siberian roe deer based on mtDNA cytochrome *b* (1,140bp). Bayesian posterior probability is shown for branches with over 50% support. Cluster A, B, C and D were identical with Bayesian tree in the Figure 1.

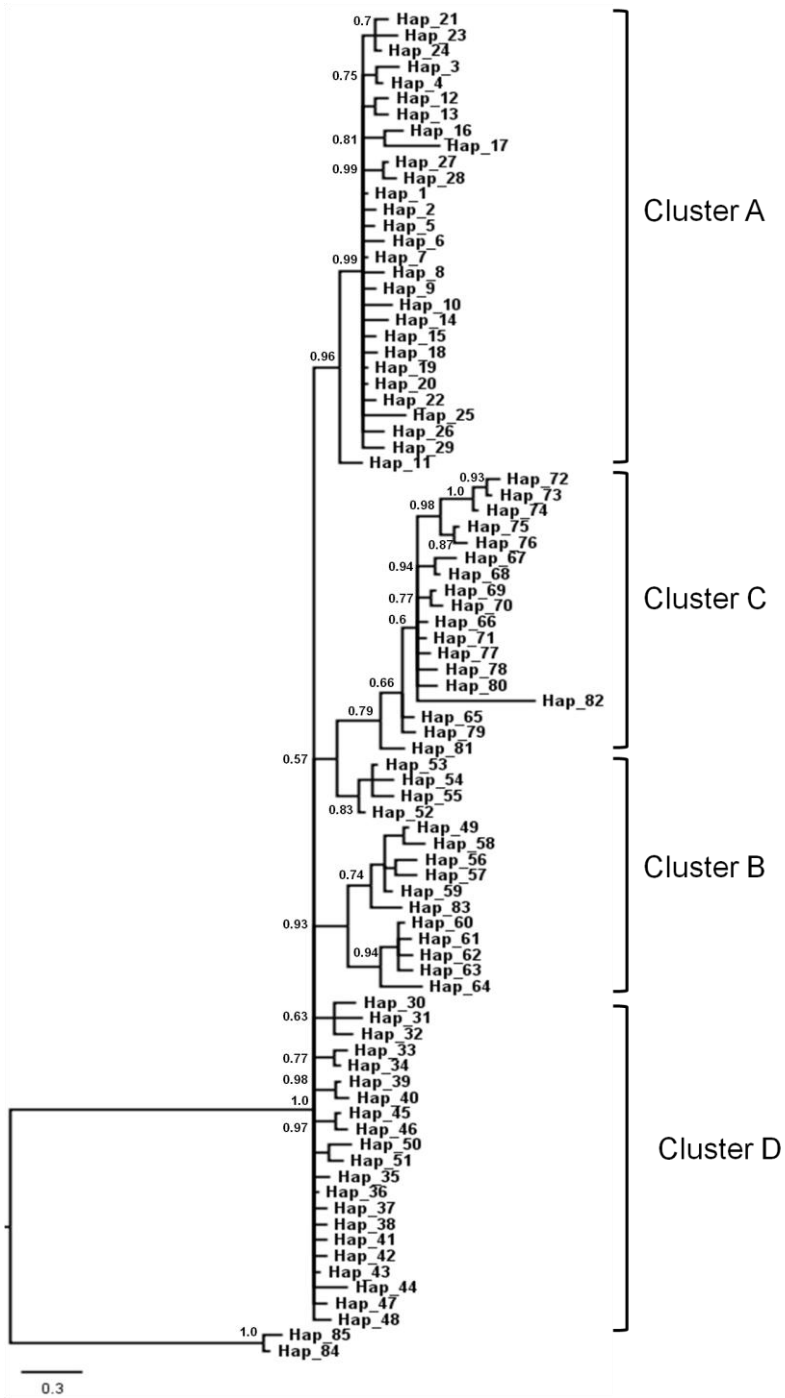


Figure 4. Bayesian (MCMC) haplotype tree of Siberian roe deer based on mtDNA control region (923bp). Bayesian posterior probability is shown for branches with over 50% support. Cluster A, B, C and D were identical with Bayesian tree in the Figure 1.

Median-joining network approach is useful for detect genealogies among interpopulation analysis (Bandelt *et al.*, 1999). Network showed the star-like shape (Figure 5), in which cluster D is positioned as a central cluster and connected to all the other clusters, cluster A, B and C. Cluster A, B and C are not interconnected from each other, but are related to the cluster D, with long branches, indicating the occurrence of large numbers of missing mutation steps.

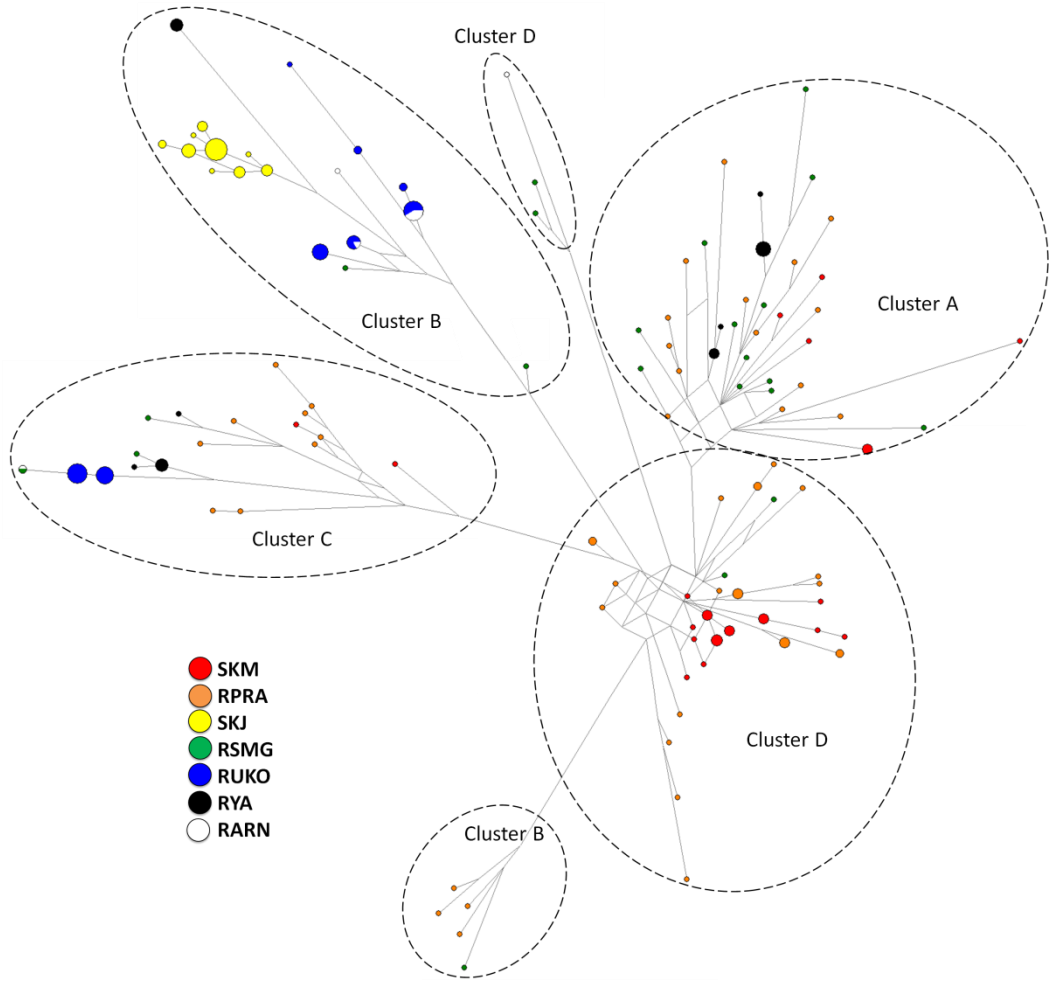


Figure 5. The median-joining network based on the haplotype data of each individual. Branch lengths are scaled to the number of nucleotide substitutions and size of circles is proportional to the haplotype frequency. Dotted lines indicate cluster of phylogeneic tree.

Genetic divergence of Siberian roe deer

Levels of population differentiation, F_{ST}, between geographic populations were ranged from 0.037 (in RPRA vs. RSMG) to 0.661 (in SKJ vs. SKM) (Table 4). Significant level ($P < 0.002$) of population differentiation was observed between South Korea, Jeju (SKJ) and the other six populations. Other populations that were significantly different from all the others were those from the western part of geographical range Urals (RUKO) and Western Siberia (RARN), however genetic difference between these two populations was not statistically significant. Genetic differences between the samples from the eastern part of *C. pygargus* geographical range (RSMG, SKM and RPRA) were not statistically significant from each other (except SKM vs. RSMG). Yakutia (RYA), one of peripheral populations, showed significant difference from most populations (SKM, RARN and RUKO), but genetically similar to Trans-Baikal region (RSMG) with non-significant genetic difference. Also, genetic difference between Yakutia (RYA) and Russian Far East (RPRA) showed low (0.109) but significant difference.

Table 4. Pairwise estimates of genetic differentiation between roe deer populations. See Table 1 for regional abbreviation.

	SKJ	SKM	RPRA	RYA	RSMG	RARN	RUKO
SKJ	–	*	*	*	*	*	*
SKM	0.661	–	NS	*	*	*	*
RPRA	0.519	0.054	–	*	NS	*	*
RYA	0.591	0.203	0.109	–	NS	*	*
RSMG	0.588	0.106	0.037	0.040	–	*	*
RARN	0.637	0.413	0.287	0.252	0.232	–	NS
RUKO	0.528	0.382	0.302	0.218	0.261	0.131	–

Population pairwise F_{ST} are below the diagonal. P value is carrying out by Bonfferoni correction (*$P < 0.002$, NS: not significant).

Demographic expansion of Siberian roe deer

Different tests for demographic fluctuations in Siberian roe deer showed various aspects of population growth for roe deer population. Analysis of mismatch distributions (Figure 6) have shown signature of recent demographic growth for populations from eastern part (SKM and RPRA) of geographic range, South Korea, Jeju (SKJ) and Trans-Baikal and Mongolia (RSMG). For these groups, both Tajima D and Fu's *Fs* in neutrality tests showed negative values, and especially Fu's *Fs* for RPRA and RSMG were significant from the expected under the hypothesis about the recent demographic expansion (Table 5).

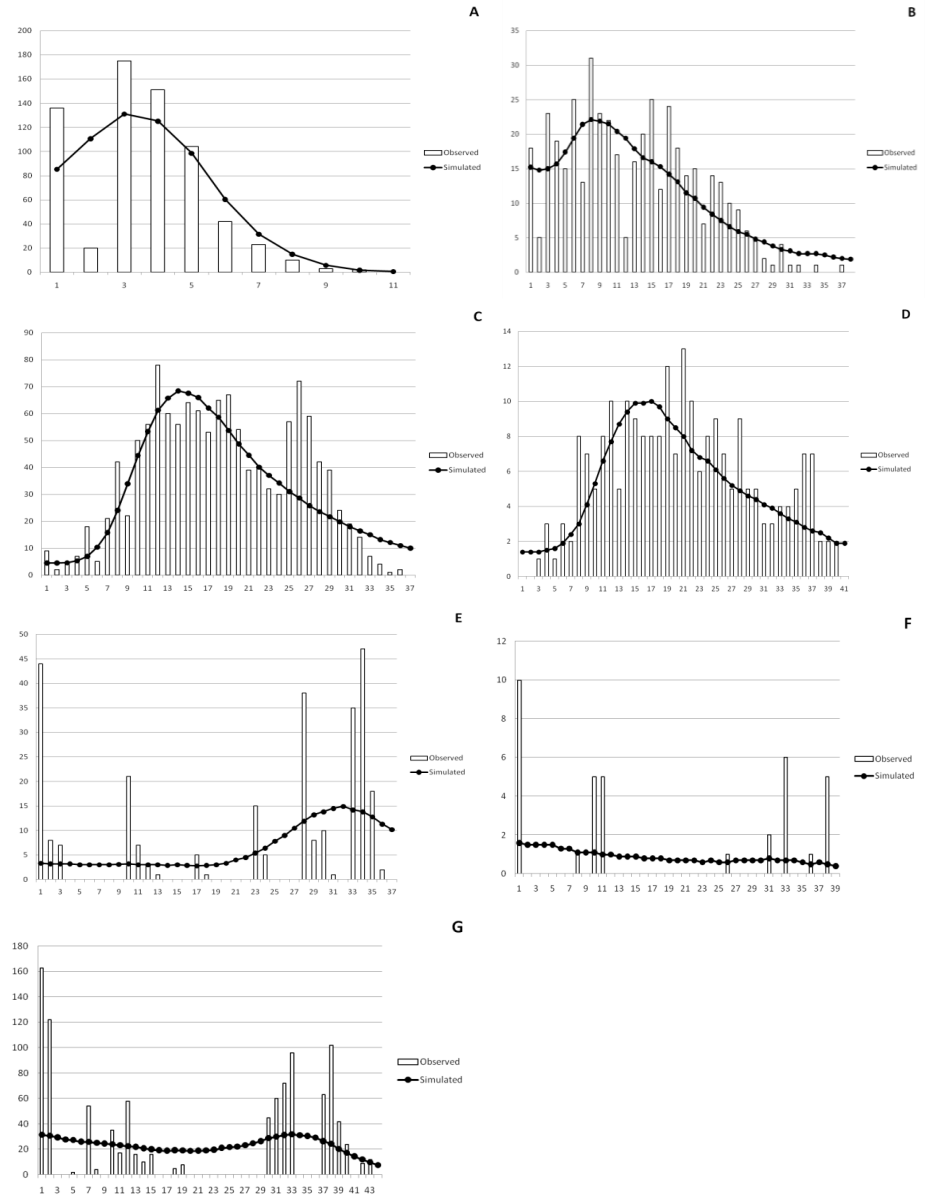


Figure 6. Mismatch distribution of each regional population under the sudden expansion model. Mismatch distributions based on pairwise site differences between sequences. The expected curve (solid line with dot) was obtained from simulated values computed from the data under the model of demographic expansion. (A) SKJ; (B) SKM; (C) RPRA; (D) RSMG; (E) RYA; (F) RARN; (G) RUKO. See Table 1 for regional abbreviation.

Table 5. Tests for demographic fluctuations in each region of Siberian roe deer. See Table 1 for regional abbreviation.

Population	N	D (*P*–value)	*Fs* (*P*–value)	r
SKJ	37	−0.248 (0.45)	−0.816 (0.39)	0.101 (0.101)
SKM	30	−1.265 (0.08)	−2.143 (0.23)	0.011 (0.751)
RPRA	51	−1.093 (0.13)	−20.88 (0.00)*	0.003 (0.548)
RYA	24	1.416 (0.95)	10.25 (0.99)	0.096 (0.000)
RSMG	22	−1.130 (0.12)	−8.801 (0.00)*	0.006 (0.943)
RARN	9	−1.006 (0.15)	4.694 (0.97)	0.221 (0.053)
RUKO	46	2.611 (0.99)	18.27 (1.00)	0.046 (0.006)
Total	219	−0.102 (0.41)	0.082 (0.50)	–

D, Tajima D; *Fs*, Fu's *Fs* (*$P < 0.05$); r, raggedness value (*P*–value in parenthesis) from mismatch analysis.

Discussion

Genetic diversity and demographic history

In this study, we investigated and compared genetic parameters for populations from different parts of the geographical range of *Capreolus pygargus*. Samples from Western Siberia (RARN) and Trans-Baikal region (RSMG) could be treated as taken from the central part of the species modern distribution; Russian Far East (RPRA) and South Korea (SKM) represent the eastern part of it. Sample from Jeju Island (SKJ) represents the isolated population. Siberian roe deer in Yakutia (RYA) is situated at the northern periphery of the species range. Ural Mountains (RUKO) it forms sympatric populations with European roe deer (*Caprolus capreolus*) (Danilkin, 1999; Zvychaynaya *et al.*, 2013). Thus, Ural region can be treated as situated close to the western periphery of geographical range of *C. pygargus*.

Relative comparison of genetic diversity estimates among other species would be informative to understanding of the present genetic status of Siberian roe deer. Genetic diversity of control region (Table 2) was only compared with previous studies due to many former studies of Siberian and European roe deer (intraspecific) were published based on an analysis of control region. Most of Siberian roe deer populations revealed similar levels of haplotype and nucleotide diversity in control region (Hd= 0.722-0.984 except SKJ, π= 0.935-1.261% except SKM, SKJ), compared

to those previously reported for Siberian roe deer (Hd= 0.93, π= 1.2%; Randi *et al.*, 1998), (Hd= 0.872, π= 1.1%; Xiao *et al.*, 2007), (Hd= 0.98, π= 1.66%; Vorobieva *et al.*, 2011), (Hd= 0.943, π= 1.1%; Lorenzini *et al.*, 2014). Also, moderate level of genetic diversity were observed in the most of Siberian roe deer populations, compared to other Cervidae such as European roe deer (Hd= 0.93, π= 1.1%; Randi *et al.*, 1998), (Hd= 0.971, π= 1.1%; Randi *et al.*, 2004), (Hd= 0.942, π= 0.95%; Lorenzini *et al.*, 2014), sika deer (*Cervus nippon*) (Hd= 0.932, π= 1.06%; Wu *et al.*, 2004), Eld's deer (*C. eldi*) (Hd= 0.81 – 0.89, π= 1.4–2.4%; Balakrishnan *et al.*, 2003) and reindeer (*Rangifer tarandus*) (Hd= 0.987, π= 1.8%; Kholodova *et al.*, 2011).

Grant and Bowen (1998) suggested the interpretation of differences between haplotype and nucleotide diversities as a means of assessing the demographic history of populations. Especially, roe deer from Jeju Island, South Korea (SKJ) showed the lowest level of genetic diversity among Siberian roe deer and compared with other species. This presumably is due to the geographic isolation and small founder on Jeju Island. Roe deer inhabited in Jeju Island during the last glacial maximum (LGM) when there was a bridge between the island and the Korean peninsula. It is probable that a relatively small group of animals was founded in the island after the last glacial periods, which led to reduced genetic diversity due to processes such as founder effect and genetic drift.

The Mainland Korea (SKM) showed the relatively high

haplotype diversity and low nucleotide diversity among the populations. This is attributed to rapid population expansion after a period of low effective population size by bottleneck (Grant and Bowen, 1998). This population expansion was also indicated in the mismatch distribution (Figure 6), but non-significant negative values in the Tajima D, Fu's *Fs* (Table 5).

Both high level of genetic diversity (RYA and RSMG) attributed mixed samples from historically split populations or stable populations with large long-term effective population sizes (Grant and Bowen, 1998). The northern periphery of geographical range in Yakutia (RYA) belongs to former case. This could be related to history of the population previously it was hypothesized that roe deer population in Yakutia originated from two subspecies, *C. p. pygargus* and *C. p. tianschanicus* (Boeskorov and Danilkin, 1998; Argunov, 2013). Co-occurrence of two subspecies therefore could be a reason for high level of genetic diversity in a given sample. Also, moderate level of genetic diversity (RPRA, RARN and RUKO) attributed relatively stable populations.

Phylogenetic relationships in Siberian roe deer

Phylogenetic trees and median-joining network revealed two main trends in genetic relationships between the samples. First, we cannot treat any of the haplogroups as ancestral to the others. Though cluster D occupies basal position in the trees (Figure 1) and center of the star-like shape in the median-joining network (Figure 5), but it is more likely that all haplogroups separated from

the common ancestor at approximately the same time (Pleistocene, 0.7–0.4 MYA). Position of the haplogroups in the median-joining networks suggests that all samples' haplotypes and haplogroups could originate from some basal haplotypes, which are not presented in the sample under this study. Cluster D is probably the closest to this ancestral group, while groups A–C changed significantly since the time of divergence from this putative ancestor.

Geographical distribution of the haplogroups (Figure 2) indicates that populations to east from Lake Baikal are genetically different pattern from those in the central and western Siberia and in Urals region. Particularly, eastern populations are marked with haplogroups A and D, which are small or not presented in "western" regions. Data from South Siberia presented by Zvychainaya *et al.* (2011) showed that roe deer from Central Siberia (Krasnoyarsk, Irkutsk, Buryatiaand Tuva) are genetically more similar to roe deer from the western part of species range (Urals and Western Siberia), than to roe deer inhabiting Russian Far East and Yakutia. According to Zvychainaya *et al.* (2011), there are two main haplogroups found in the area from Urals to Baikal. These groups are probably similar to haplogroups B and C in our study, while only one haplogroups found described by Zvychainaya *et al.* (2011) for the area from Lake Baikal to Pacific Ocean look similar to haplogroups A or D in this study. Similar to our results, the lake Baikal is a region where "eastern" and "western" haplogroups could be found together.

There are several possible scenarios that could explain

observed distribution of genetic lineages. One possible scenario is that all the analyzed samples originated from the same ancestral group, which was preserved in some refugia during the periods of climate change in Pleistocene. Possible geographical locations of the putative ancestral group are mountains of the southern Siberia, particularly Altay, Tyan-Shan and Sayan mountains. This suggestion is supported by distribution of cluster D, which is presented only in the central-eastern part of the species geographical range and also by the fact that all four haplogroups are presented only in the sample from Trans-Baikal and Northern Mongolia (RSMG) and Russian Far East (RPRA). On the other hand, this suggestion contradicts the fact that Zvychainaya *et al.* (2011) did not find eastern haplotypes in the central Siberia. However, this could result from a relatively small number of samples (total 20 samples for 4 regions of Southern Siberia and Kazakhstan) or the refugia situated to the south from the sampled areas.

Another possible scenario of the observed diversity of haplogroups in various parts of *C. pygargus* geographical range is that there were several genetic lineages which diverged independently from common ancestor and could be isolated from each other in Pleistocene during the periods of formation of the big open spaces in Central Asia followed by periods of glaciations (Matjushkin, 1982). In this case high diversity of haplotypes and haplogroups in the Trans-Baikal region (RSMG) and Russian Far East (RPRA) are the result of secondary colonization of this area by animals from different areas after the periods of climate change.

This fits to the results of phylogenetic tree with formation of four main haplogroups from independent lineages and mismatch analysis showing signs of recent demographic growth in the populations of South Siberia (RSMG) and Amur region (RPRA). Supposedly, more intensive sampling of the regions of southern Siberia and Kazakhstan could reveal roe deer populations with more diverse genetic composition and help finding the haplotypes ancestral to those described in this study.

The second important and very intriguing trend is the genetic composition of the isolated population in Jeju Island (SKJ). Jeju Island (SKJ) was indeed composed of only one haplogroup (cluster B) and indicating somehow homogeneous genetic composition. Migration of roe deer to the Jeju Island could take place only during the periods of glaciation when the island was connected to continent. Obtained results also shed light on the taxonomic status of the roe deer inhabiting Jeju Island, however study of B-chromosome is necessary to classification of Jeju Island population (SKJ) as a subspecies. Genetically distinct of this population (Table 4) to roe deer from the all other population does not allow to treat Jeju roe deer as *C. p. tianschanicus* (Koh *et al.*, 2000), neither as distinct subspecies as suggested (Koh and Randi, 2001; Park *et al.*, 2014). Siberian roe deer from Jeju Island are indeed different from the roe deer from mainland Korea (Lee *et al.*, 2015) but they not appeared to be distinct phylogenetic clade and distributed main haplogroup of western population. On the other hand, Jeju Island roe deer are much smaller than those, inhabiting the western part of the range,

particularly the total body length and height in shoulder are almost 1.5 times smaller (144 vs 96 cm and 92 vs 57.5 cm respectively) in Jeju roe deer (Danilkin, 1999; Park *et al.*, 2011). This phenomenon is presented not only jeju Island but also all Siberian roe deer.

The taxonomic status of the Siberian roe deer did not clearly observe in the phylogenetic tree as a clade. Genetic similarity associated with obvious morphological differences gives an example of discordance between genetic and morphological evolution in mammals. Typically this problem is discussed for so called "cryptic species", the taxa which cannot be distinguished by morphological traits, but differ genetically (Bickford *et al.*, 2007). In case of the Siberian roe deer we observe the opposite morphologically different populations belong to the same genetic lineage. Such trend was previously found on the highest taxonomic levels, particularly, elephants were found to be genetically similar to such biologically dissimilar groups, as dugong, elephant shrews, tenrecs, golden moles and aardvark. The common descent of the elephants with a group of marine mammals and many smaller enigmatic African placental mammals is now broadly accepted and Afrotheria is considered as one of four mammalian superorders (Kuntner *et al.*, 2011). On the within-species level incoherence of genetic and morphological traits was demonstrated for small mammals (Smirnov and Fedorov, 2003). In our case lack of correlation between genetic and morphological traits is clearly related to the type of molecular marker mitochondrial DNA, because comparison of populations based on microsatellites revealed clear

differences between Jeju population and roe deer from the western part of the geographical range (Lee *et al.*, 2015).

Finally, our data show that roe deer in the area from Ural to Pacific Ocean is not clearly described ranges of subspecies and phylogeograpic distribution pattern. But, Siberian roe deer have four haplogroups, also various haplogroup exist in the east Siberia regions and two haplogroups mainly exist in the west Siberia regions. Population of Siberian roe deer on Jeju Island is a unique one where conservation of one of the ancient mitochondrial lineages is coupled with specific morphological features.

CHAPTER Ⅱ.

Genetic diversity and genetic structure of the Siberian roe deer (*Capreolus pygargus*) populations from Asia

Introduction

The family Cervidae is widely distributed throughout Eurasia and includes 40 species of deer (Bouvrain *et al.*, 1989). The roe deer (*Capreolus* Gray, 1821) is one of the most widespread meso-mammals in Cervidae and includes two species, the smaller European roe deer (*C. capreolus* Linnaeus, 1758) and the larger Siberian roe deer (*C. pygargus* Pallas, 1771). The two species of deer are distinguished mainly by differences in morphology and karyotype. The Siberian roe deer is distributed in the Palaearctic throughout continental Asia (Danikin, 1996) and some parts of Eastern Europe (Matosiuk *et al.*, 2014). Although the classification of subspecies is still controversial, it is widely accepted that the Siberian roe deer comprises of at least two or three subspecies, *C. pygargus pygargus* (from Volga river to Lake Baikal and Northeastern Russia), *C. pygargus tianschanicus* (or *C. c. bedfordi* Thomas, 1908) (Tianshan mountain, Mongolia, Russian Far East and Korea) and *C. pygargus melanotis* Miller, 1911 (Eastern Tibet, and Gansu and Sichuan Province, China).

For mammal species such as Siberian roe deer, which is distributed across extensive geographical range, contemporary level of genetic variation and population structure may be shaped by interaction of both natural and anthropogenic factors (Hewitt, 2000; Segelbacher *et al.*, 2010). Especially numerous human activities, such as habitat destruction / fragmentation, hunting, and human-mediated translocation, have influenced distribution, population structure, and genetic diversity of natural wildlife during the last few centuries (Breitenmoser, 1998; Harris *et al.*, 2002). Fossil records report that Siberian roe deer territory was once connected to the northern Caucasus (Korotkevich and Danilkin, 1992). However, population size drastically diminished supposedly because of overhunting in Western Siberia and Northeastern Siberia during the 19th and 20th centuries (Danilkin, 1995). Regardless, the original historic distribution has almost completely recovered.

Population genetics and phylogeography of European roe deer have been well studied (Lorenzini *et al.*, 2002; Vernesi *et al.*, 2002; Lorenzini *et al.*, 2003; Randi *et al.*, 2004; Lorenzini and Lovari, 2006; Royo *et al.*, 2007; Kamieniarz *et al.*, 2011; Baker and Hoelzel, 2013; Lorenzini *et al.*, 2014). Most studies using mitochondrial and nuclear markers for European roe deer revealed geographic pattern in the population structure, with generally high levels of genetic variation. The Siberian roe deer is relatively less studied and most of the genetic studies of the species have been obtained from phylogenetic inferences using mitochondrial DNA sequence data. These studies using mtDNA demonstrated that Siberian roe deer

can be divided into several major clusters with geographic patterns; the cluster in eastern Siberia and the western Siberia (Randi *et al.*, 1998; Zvychainaya *et al.*, 2001). In contrast, some phylogeographic studies have reported no apparent geographic pattern of genetic variation among the broadly sampled Siberian roe deer (Sheremetyeva *et al.*, 2010; Lorenzini *et al.*, 2014).

Overall, population boundaries and the genetic structuring of the Siberian roe deer remain unclear and the classification of *C. pygargus* subspecies is still under debate. Although phylogenetic studies using mtDNA sequences provided valuable information regarding the genetic relationship and phylogeographic inferences of the Siberian roe deer, studies on population genetics using the fast-evolving nuclear makers, such as microsatellites, can provide additional information to better understand the present status of genetic diversity and population structure of geographic Siberian roe deer in Asia.

In this study, we investigated microsatellite variability for Siberian roe deer collected throughout Asia to examine the level of population genetic structure and the amount of genetic variation of Siberian roe deer. These data were applied to discuss how historical and demographic dynamics have affected the recent and past population genetic structure of Siberian roe deer.

Materials and Methods

Sample collection and DNA extraction

A total of 189 individuals of *C. pygargus* were collected from ten locations in Russia, Mongolia and South Korea (Appendix S1). SKJ: South Korea, Jeju (N= 33), SKM: South Korea Mainland (N= 31), RPR: Russia, Primorsky Krai (N= 30), RYA: Russia, Yakutia (N= 18), RSO: Russia, Sokhondinsky (N= 9), MGN: Mongolia, Northern part (N= 12), RAL: Russia, Altaisky Krai (N= 5), RNO: Russia, Novosibirsk Oblast' (N= 7), RUR: Russia, Ural (N= 23), RKU: Russia, Kurganskaya Oblast' (N= 21). This experimental work was conducted with permission by the Conservation Genome Resource Bank for Korean Wildlife (CGRB) that provided the roe deer samples for this study. All samples were legally collected and deposited into CGRB. The procedures involving animal samples followed the guidelines by Seoul National University Institutional Animal Care and Use Committee (SNU IACUC). Tissue (muscle, skin and liver) and blood samples were collected across the current distribution range of *C. pygargus* from 2001 to 2011, and were frozen at −70 ℃ deep freezer in the CGRB or stored in ethanol until DNA extraction. Genomic DNA was extracted from individual sample using the DNeasy tissue and blood kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

Microsatellite analysis

A total of 12 microsatellite loci were used and tested for genotyping and genetic analysis of *C. pygargus* sampled. Microsatellite markers previously developed from rein deer (RT1, RT20, RT23, RT24, RT30), cattle (MB25, BM757, CSSM41, IDNGA8, IDNGA29), and European roe deer (Roe01, Roe09) have proved to be polymorphic in Siberian roe deer, and were used through the cross-species amplification in this study. Genomic DNA was amplified for genotyping under the following conditions. The touchdown profile for the PCR amplification was at 94 ℃ for 15 min, followed by 20 cycles at 94 ℃ for 30 S, 65 ℃ for 60 S, and 72 ℃ for 30 S, with annealing temperature decreased by 0.5 ℃ per cycle to 55 ℃. The touchdown cycles were followed by an additional 25 cycles at 94 ℃ for 30 S, 55 ℃ for 1 min, 72 ℃ for 30 S, and a final extension at 72 ℃ for 20 min. The PCR reaction mixture contained $MgCl_2$ (2 mM), dNTP (each 0.2 mM), and i-Star *Taq* DNA polymerase (0.025 U) of iNtRON biotechnology Inc (Korea). One of three (Hex, 6-Fam, Tamra) fluorescently-labeled M13 primers (0.26 pmol), unlabeled M13-tailed forward primer (0.13 pmol), and reverse primer (0.26 pmol) were also added to the reaction tubes. All amplifications were implemented in a volume of 15 μl in TaKaRa thermal cyclers. Alleles were determined by ABI Prism3730 XL DNA Analyzer (Applied Biosystemsinc, USA) using GENESCAN-500 [Rox] size standard and analyzed GeneMapper version 3.7 (Applied Biosystemsinc, USA)

Data analysis

Ten locations were used for basic analyses to obtain the summary statistics, and to improve statistical power for certain analysis like Bottleneck test, six locations with geographical proximity and small sample size were further pooled into three locations such as, (RSMG: RSO & MGN), (RARN: RAL & RNO) and (RURK: RUL & RKU). The number of all alleles per locus and population (MNA), observed heterozygosity (H_O) and expected heterozygosity (H_E) in Hardy–Weinberg equilibrium were estimated for each locus using the Microsatellite Toolkit, version 3.0 (Park, 2001). Allelic richness (*Ar*), *F*–statistics ($F_{IS,}$ F_{ST}) (Weir and Cockerham, 1984) and genotype linkage disequilibrium for all pair of loci in population were determined using the program FSTAT, version 2.9.3 (Goudet, 1995). Allelic Richness is one of important measures of genetic diversity and is calculated based on a minimum sample size of each population to compensate for the differences in sample size among populations. Wilcoxon signed rank test was employed to assess differences in allelic richness and expected heterozygosity that are corrected by small sample sizes using the STATISTIX version 8.1 (Analytical Software, Statistix; Tallahassee, FL, USA, 2000). The number of loci with null alleles was assessed using MICRO–CHECKER (Van Oosterhout *et al.*, 2004). Occurrence of null alleles can lead to diminution in genetic diversity and inflate genetic differentiation among population (Dakin and Avise, 2004). Null alleles can be common owing to ascertainment bias and sequence variation especially when microsatellites from cross–species

amplification are used. The number of private alleles and genetic characteristics of 12 microsatellite loci for ten regional samples were determined using the GenAlEx version 6.1 (Peakall and Smouse, 2006). The program CERVUS, version 2.0 was used to calculate the polymorphism information content (PIC), observed heterozygosity (H_O) and expected heterozygosity (H_E) of each locus (Kalinowski *et al.*, 2007). Deviations from Hardy–Weinberg equilibrium (HWE) for each geographic population were evaluated using the exact probability test (Guo and Thompson, 1992) using the Fisher procedure calculated by GENEPOP version 3.3 (Raymond and Rousset, 1995).

Gene flow measures

The pattern of gene flow between populations was measured using two different approaches. First, the effective number of migrants per generation (N_em) between populations was calculated from with the following formula: $N_em = (1 - F_{ST}) / 4F_{ST}$ (Wright, 1931), where N_e is the effective population size and m is the migration rate. This gene flow (N_em) estimate is an approximation of a particular theoretical model (Island model) at equilibrium that migration occurs at the same rate with equal population size. F_{ST} is a measure of genetic differentiation between populations and allows estimation of relatively long–term gene flow based on allele frequency distributions. Pairwise F_{ST} between populations and their significance calculated using the program FSTAT version 2.9.3 (Goudet, 1995). Also, pairwise F_{ST}'s were corrected by the ENA

method (excluding null alleles) using the FREENA software (Chapuis and Estoup, 2007). The difference between the ENA corrected and uncorrected F_{ST} values was evaluated by the Wilcoxon rank sum test using the STATISTIX version 8.1 (Analytical Software, Statistix; Tallahassee, FL, USA, 2000).

Genetic relationship

The genetic relationship between populations was evaluated by the Nei's genetic distances (D_A) (Nei *et al.*, 1983) based on allele frequencies using the program DISPAN (Ota, 1993). Genetic relationship trees were constructed by unweighted pair group method with the arithmetic mean (UPGMA) (Sneath and Sokal, 1973) based on D_A distance with 1000 bootstrap replications to test the validity of tree topologies. Principal coordinate analysis (PCA) was conducted using the covariance matrix of allele frequencies using the GENALEX version 6.1 (Peakall and Smouse, 2006). Two principal values with the first and second highest factor scores were employed to construct a scatter diagram to visualize genetic relationships among populations. The GENALEX version 6.1 was further used to carry out hierarchical analysis of molecular variance (AMOVA) of genetic differentiation among populations and regions, and F–statistics (F_{RT}, F_{SR}, F_{ST}, F_{IS} and F_{IT}). According to the geographical distance, ten roe deer populations were divided into four main regions for the AMOVA analysis: Jeju Island, South Korea (SKJ), East region (SKM, RPR), Central region (RYA, RSO, MGN) and West region (RAL, RNO, RUL and RKU). Besides, according to

the structure result (three clusters), eight roe deer populations were divided into three main regions excluding the two admixed populations (RYA, RAL) for the AMOVA analysis: Jeju Island, South Korea (SKJ), Eastern region (SKM, RPR, RSO and MGN) and Western region (RNO, RUL and RKU). Additionally, seven populations were divided into two main regions with SKJ and two admixed populations (RYA and RAL) excluded: Eastern region (SKM, RPR, RSO, MGN) and Western region (RNO, RUL, RKU). Significance level was calculated by the permutation procedure (999 permutations).

Population structure

Existence of population genetic structuring was evaluated using the model-based Bayesian clustering method in the program STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000), which infers the number of genetic clusters (K) without prior information about population origin. This method calculates independent assessments of each individual for each cluster. The log-likelihood data [Ln Pr (X/K)] was estimated for given K between 1 and 10 with ten independent runs set by 1,000,000 Markov chain Monte Carlo (MCMC) iterations followed by burn-in period of 100,000 iterations. The "real" value of K within the dataset was estimated from the Ln Pr (X/K) according to the method of Evanno *et al.* (2005) in which log-likelihood values, and variance from each replicate of K were used to calculate ΔK. An *ad hoc* statistic test in this parameter was used in simulations to identify the true number of genetic clusters, which offers accurate means to selecting K instead of choosing a K with

the highest log probability that could lead to overestimated K (Evanno *et al.*, 2005). Existence of Isolation-by-distance (IBD) (Wright, 1931) was obtained by the regression of genetic distance (F_{ST} / (1-F_{ST})) on geographic distance (Ln-Km) between pairs of populations. The correlations for two variables and probability were carried out using the Mantel's test in GENALEX version 6.1 and significance was determined based on 999 permutations (Peakall and Smouse, 2006).

We also applied Monmonier's maximum difference algorithm to highlight geographical features with obvious genetic discontinuity between populations using the program BARRIER version 2.2 (Manni *et al.*, 2004). The data from nine populations except Jeju Island, Korea (SKJ) were analyzed to detect putative barriers of gene flow among the populations. Geographical coordinates were used for each population and connected by Delauney triangulation using a pairwise F_{ST} genetic matrix. We conducted the analysis using F_{ST} for each of the eleven microsatellite loci; exclude IDVGA29 due to low polymorphism, to make sure that the barriers were not verified with strong differentiation at only few loci. Each locus indicates how many support a given barrier and putative genetic boundaries were identified across the geographical landscapes. Pairwise F_{ST}, R_{ST} and pR_{ST} (R_{ST} computed after allele size permutation test with 1000 randomizations) were calculated per each population and locus to estimate the main causes of population differentiation in Siberian roe deer using program

SPAGeDi (Hardy and Vekemans, 2002; Hardy *et al.*, 2003). R_{ST} was compared against the distribution of pR_{ST} values.

Bottleneck detection

Three different approaches were used to detect molecular evidence of historical population bottleneck. First, we tested for deviations of expected heterozygosity (*H*e) relative to heterozygosity expected at drift−mutation equilibrium (H_{eq}) by Wilcoxon sign−rank tests ($\propto$ = 0.05, $\propto$ = 0.01) (Luikart *et al.*, 1998a) using the BOTTLENECK version 1.2.02 (Cornuet and Luikart, 1996; Piry *et al.*, 1999). During bottlenecks, the number of rare alleles is reduced faster than the heterozygosity at polymorphic loci due to drift (Nei *et al.*, 1983). Thus the bottleneck test can detect this disparity when H_e becomes larger than H_{eq}, because H_{eq} reflects allele number and sample size. We used a two−phase mutation model (TPM) (Di Rienzo *et al.*, 1994) using a setting of 10% multiple−step mutations and 90% single−step mutations with 1,000 iterations. Secondly, we checked out a mode−shift in distributions of allele frequencies from the L−shaped distribution under the mutation−drift equilibrium, expecting distorted distribution under the recent population bottleneck (Luikart *et al.*, 1998b).

Lastly, *M* value of Garza and Williamson's (2001) was calculated for each population to detect the long−term decrease of population size using the program AGARST version 3.3 (Harley, 2001). *M* is the mean ratio of the total number of alleles to the range of allele size. This test is useful for detecting a bottleneck further in the past (> 100 generations). Meta−analysis for natural populations revealed

that historically reduced or founded populations had M−ratio < 0.68, but stable populations showed M > 0.82.

Results

Genetic variability of Siberian roe deer

Genetic characteristics of 12 microsatellite loci from Siberian roe deer sampled at each location are shown in Table 6. Source information and characteristics of 12 microsatellite loci from other species are shown in Table 7. A total of 122 alleles were detected for 189 individuals of ten Siberian roe deer populations. The number of alleles per locus varied from 2 (BM25) to 24 (MB757) with a mean of 10.17. Microsatellite loci showed various levels of polymorphism, with the polymorphism information content (PIC) values ranging from 0.062 (IDVGA29) to 0.926 (BM757). Most loci, except IDVGA29, showed moderate to high polymorphism.

Table 6. Genetic characteristics of 12 microsatellite loci for Siberian roe deer from seven geographic regions in Asia

Locus	SKJ	SKM	RPR	RYA	RSMG	RARN	RURK	Mean
RT1								
No. of alleles	1	13	13	9	15	6	9	9.429
H_O	0.000	0.742	0.607	0.882	0.667	0.750	0.698	0.621
H_E	0.000	0.871	0.893	0.815	0.907	0.785	0.800	0.724
HWE P-value	NA	0.0106	0.0000	0.4639	0.0000	0.2227	0.0010	-
RT20								
No. of alleles	4	4	5	5	4	4	5	4.429
H_O	0.107	0.484	0.483	0.556	0.667	0.667	0.690	0.522
H_E	0.427	0.648	0.729	0.739	0.676	0.740	0.642	0.657
HWE P-value	0.0000	0.1252	0.0027	0.0463	0.6315	0.3087	0.2435	-
RT23								
No. of alleles	4	4	5	1	4	3	3	3.429
H_O	0.545	0.419	0.517	0.000	0.524	0.250	0.477	0.390
H_E	0.606	0.563	0.467	0.000	0.422	0.226	0.428	0.388
HWE P-value	0.2140	0.0056	0.8054	NA	1.0000	1.0000	0.6717	-
RT24								
No. of alleles	3	5	5	5	4	4	5	4.429
H_O	0.303	0.667	0.600	0.788	0.571	0.750	0.773	0.635
H_E	0.326	0.669	0.721	0.719	0.670	0.736	0.655	0.642
HWE P-value	0.4697	0.4372	0.2165	0.3950	0.2238	0.8595	0.2946	-

Table 6. (Continued)

Locus	SKJ	SKM	RPR	RYA	RSMG	RARN	RURK	Mean
RT30								
No. of alleles	4	15	18	10	12	4	8	10.143
H_O	0.758	0.645	0.833	0.765	0.900	0.500	0.705	0.729
H_E	0.549	0.898	0.911	0.858	0.823	0.663	0.773	0.782
HWE P-value	0.0551	0.0000	0.0305	0.3283	1.0000	0.1733	0.0012	-
Roe01								
No. of alleles	3	3	3	3	3	2	2	2.714
H_O	0.333	0.516	0.433	0.706	0.381	0.833	0.886	0.584
H_E	0.282	0.398	0.346	0.503	0.316	0.500	0.500	0.407
HWE P-value	1.000	0.2582	0.4458	0.2104	1.0000	0.0764	0.0000	-
Roe09								
No. of alleles	2	2	2	2	4	2	2	2.286
H_O	0.531	0.194	0.633	0.188	0.619	0.364	0.182	0.387
H_E	0.500	0.458	0.499	0.482	0.517	0.397	0.499	0.479
HWE P-value	1.0000	0.0016	0.2705	0.0303	0.8623	1.0000	0.0000	-
MB25								
No. of alleles	2	2	2	2	2	2	2	2.000
H_O	0.030	0.161	0.233	0.333	0.190	0.500	0.205	0.236
H_E	0.030	0.398	0.499	0.346	0.499	0.444	0.283	0.357
HWE P-value	NA	0.0018	0.0037	1.0000	0.0068	1.0000	0.0857	-

Table 6. (Continued)

Locus	SKJ	SKM	RPR	RYA	RSMG	RARN	RURK	Mean
BM757								
No. of alleles	7	15	18	15	17	10	12	13.429
H_O	0.576	0.839	0.867	0.625	0.810	0.833	0.841	0.770
H_E	0.641	0.908	0.920	0.906	0.922	0.875	0.828	0.857
HWE P-value	0.1089	0.0000	0.0073	0.0000	0.0529	0.0000	0.0063	-
CSSM41								
No. of alleles	7	3	3	4	4	2	2	3.571
H_O	0.281	0.290	0.200	0.364	0.286	0.167	0.114	0.243
H_E	0.437	0.350	0.413	0.318	0.255	0.375	0.312	0.351
HWE P-value	0.0000	0.2186	0.0017	1.0000	1.0000	0.0899	0.0002	-
IDVGA8								
No. of alleles	5	13	12	7	13	7	5	8.714
H_O	0.485	0.452	0.400	0.308	0.381	0.417	0.295	0.391
H_E	0.624	0.878	0.885	0.737	0.906	0.698	0.548	0.753
HWE P-value	0.1088	0.0000	0.0000	0.0017	0.0000	0.0004	0.0000	-
IDVGA29								
No. of alleles	3	1	3	1	2	1	4	2.143
H_O	0.000	0.000	0.069	0.000	0.000	0.000	0.071	0.020
H_E	0.140	0.000	0.067	0.000	0.091	0.000	0.070	0.053
HWE P-value	0.0004	NA	1.0000	NA	0.0244	NA	1.0000	-

Table 7. Source information and characteristics of 12 microsatellite markers obtained from cross-species amplification.

Locus	Size range	NA	H_E	H_O	PIC	Origin	GenBank Accession no.	Reference
RT1	221-259	18	0.890	0.587	0.877	Rein deer (*Rangifer tarandus*)	U90737	Wilson *et al.* (1997)
RT20	235-245	6	0.767	0.509	0.724	Rein deer (*Rangifer tarandus*)	U90744	Wilson *et al.* (1997)
RT23	200-208	5	0.494	0.433	0.459	Rein deer (*Rangifer tarandus*)	U90745	Wilson *et al.* (1997)
RT24	210-220	6	0.766	0.622	0.722	Rein deer (*Rangifer tarandus*)	U90746	Wilson *et al.* (1997)
RT30	201-249	22	0.914	0.738	0.905	Rein deer (*Rangifer tarandus*)	U90749	Wilson *et al.* (1997)
Roe01	152-160	5	0.438	0.580	0.359	Roe deer (*Capreolus capreolus*)	AF164070	Fickel and Reinsch (2000)
Roe09	195-201	4	0.503	0.378	0.381	Roe deer (*Capreolus capreolus*)	AF166358	Fickel and Reinsch (2000)
MB25	219-221	2	0.501	0.201	0.375	Cattle (*Bos taurus*)	-	Kappes *et al.* (1997)
BM757	170-220	24	0.933	0.775	0.926	Cattle (*Bos taurus*)	G18473	Kappes *et al.* (1997)
CSSM41	124-150	10	0.502	0.227	0.461	Cattle (*Bos taurus*)	U03816	Slate *et al.* (1998)
IDVGA8	229-259	16	0.887	0.391	0.875	Cattle (*Bos taurus*)	Z27074	Slate *et al.* (1998)
IDVGA29	155-163	4	0.063	0.029	0.062	Cattle (*Bos taurus*)	X85048	Slate *et al.* (1998)

H_E, Expected heterozygosity; H_O, Observed heterozygosity; Number of alleles (NA) and PIC were obtained from analysis for 189 Siberian roe deer

Private alleles were observed in most populations except Mid-west Siberia (RAL and RNO), but all private alleles were in very low frequency ranging from 0.011 to 0.106 (Table 8). Null alleles were present at more than one locus for each population except Mid-west Siberia (RAL and RNO), but there was no evidence of a large allele drop out (Table 8). Occurrence of null alleles at each locus showed generally low frequency less than 0.10 for most of populations. However, some loci showed various range of null alleles for certain populations as follows; 0.10 for the locus RT30 (SKM), IDVGA29 (SKJ) and BM757 (RYA), 0.30 for locus CSSM41 (SKJ, RPR and RUL), MB25 (SKM, RPR and MGN), Roe09 (SKM, RYA, and RUL), RT1 (SKM, RPR and RSO) and RT20 (SKJ, RPR and RYA). The highest frequency of null allele occurrence was found in the locus IDVGA8, with the null allele frequency of 0.60 for SKM, RPR, RSO, MGN, RKU, and RYA.

All populations showed significant deviation of observed heterozygosity from heterozygosity expected under Hardy-Weinberg equilibrium in the direction of heterozygote deficiency except Novosibirsk, Russia (RNO) (Table 8). Inbreeding coefficient (F_{IS}) estimates across all populations ranged from 0.031 to 0.247, and five populations (SKJ, SKM, RPR, RYA and RSO) were significantly deviated from zero (Table 8). Significant deviation in Hardy-Weinberg equilibrium (HWE) and F_{IS} could be due to the possibility of Wahlund effect, inbreeding (due to non-random mating or subpopulations), and/or other anomaly such as the presence of null alleles.

Measures of genetic diversity were generally high in Primorsky Krai, Russia (RPR) (mean no. of alleles per locus, MNA= 7.42, Allelic richness, Ar= 3.67, expected heterozygosity, H_E= 0.623) followed by Mainland Korea (SKM) and Northern Mongolia (MGN) (Table 8). The lowest genetic diversity was found in Jeju island, Korea (SKJ) (MNA= 3.75, Ar= 2.18, H_E= 0.386), followed by Mid-west Siberia (RAL and RNO) and West Siberia (RUL and RKU). Wilcoxon Signed Rank test revealed that allelic richness and expected heterozygosity were significantly higher in the East populations than in the West populations for the most population pairs (one tailed $p < 0.05$) (Table 9 and Figure 7).

Table 8. Genetic characteristics of Siberian roe deer in each region / location across 12 microsatellite loci.

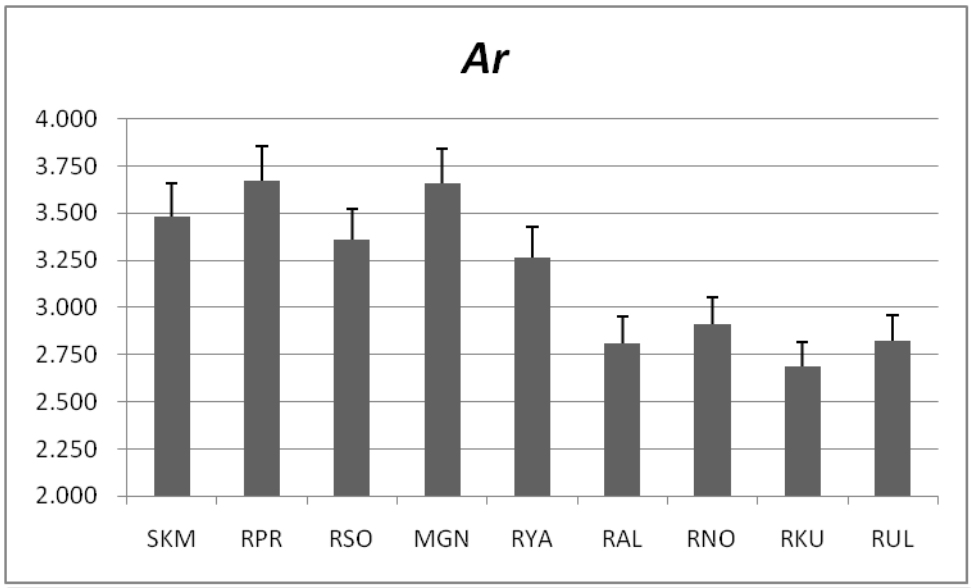
Region	N	MNA	*Ar*	H_E	H_O	F_{IS} [a]	HWE *P* [b]	Number of loci with null allele	NPA (Freq. rang)
SKJ	33	3.75	2.18	0.386	0.329	0.150*	0.000 (3)	3 (RT20, CSSM41, IDVGA29)	4 (0.016−0.106)
SKM	31	6.58	3.48	0.596	0.451	0.247*	0.000 (7)	5 (RT1, RT30, Roe09, MB25, IDVGA8)	3 (0.016−0.065)
RPR	30	7.42	3.67	0.623	0.490	0.217*	0.000 (7)	5 (RT1, RT20, MB25, CSSM41, IDVGA8)	4 (0.017−0.050)
RSMG	21	7.00	5.67	0.598	0.500	0.169*	0.000 (4)	4 (RT1, MB25, BM757, IDVGA8)	7 (0.024−0.025)
RSO	9	5.00	3.36	0.550	0.438	0.215*	0.000 (2)	2 (RT1, IDVGA8)	4 (0.056)
MGN	12	5.67	3.66	0.628	0.544	0.138 [NS]	0.000 (4)	2 (MB25, IDVGA8)	3 (0.042)
RYA	18	5.33	3.26	0.553	0.459	0.175*	0.000 (4)	4 (RT20, Roe09, BM757, IDVGA8)	5 (0.031−0.094)
RARN	12	3.92	3.87	0.560	0.503	0.107[NS]	0.000 (2)	1 (IDVGA8)	0
RAL	5	2.92	2.81	0.541	0.471	0.144 [NS]	0.003 (4)	− [c]	−
RNO	7	3.33	2.91	0.539	0.524	0.031 [NS]	0.988 (0)	− [c]	−
RURK	44	4.92	3.73	0.534	0.495	0.075[NS]	0.000 (7)	3 (Roe09, CSSM41, IDVGA8)	3 (0.011−0.012)
RKU	21	3.83	2.68	0.530	0.512	0.034 [NS]	0.000 (6)	2 (Roe09, IDVGA8)	1 (0.025)
RUL	23	4.42	2.82	0.522	0.478	0.085 [NS]	0.000 (5)	2 (Roe09, CSSM41)	2 (0.022−0.024)
Mean	27	5.56	3.68	0.550	0.461	0.163	0.000 (5)	−	−

Number of individual per population (N), Allelic diversity (MNA, mean no. of alleles per locus), allelic richness (*Ar*), expected heterozygosity (H_E) at Hardy−Weinberg equilibrium, observed heterozygosity (H_O), inbreeding coefficient (F_{IS}), and the probability (*P*) of being in Hardy−Weinberg equilibrium, null alleles, number of private alleles (NPA). [a] For F_{IS} within samples based on 2400 randomizations using the FSTAT program. [NS:] Not significant after adjusted nominal level (5%)= 0.004. [b] Probability values using the Fisher's method implemented in the GENEPOP program. Number in parentheses indicates the no. of loci showing a significant departure (P <0.05) from Hardy−Weinberg equilibrium. [c] Not determined due to small sample size.

Table 9. Wilcoxon signed rank test to assess differences in allelic richness (*Ar*) and expected heterozygosity that are corrected by small sample sizes (UH_E) (one-tailed *p*-value).

	SKJ	SKM	RPR	RYA	RSO	MGN	RAL	RNO	RUL	RKU
SKJ		0.013	0.013	0.055	0.066	0.008	0.066	0.105	0.077	0.039
SKM	0.011		0.046	0.416	0.190	0.066	0.276	0.326	0.121	0.032
RPR	0.012	0.013		0.046	0.013	0.451	0.077	0.077	0.013	0.013
RYA	0.032	0.378	0.105		0.535	0.121	0.535	0.535	0.158	0.055
RSO	0.055	0.276	0.105	0.496		0.003	0.416	0.496	0.382	0.525
MGN	0.005	0.046	0.416	0.205	0.032		0.091	0.077	0.008	0.011
RAL	0.046	0.051	0.032	0.051	0.166	0.013		0.215	0.489	0.451
RNO	0.066	0.088	0.032	0.123	0.166	0.013	0.254		0.382	0.382
RUL	0.091	0.046	0.002	0.046	0.205	0.000	0.416	0.416		0.489
RKU	0.105	0.003	0.001	0.021	0.256	0.000	0.158	0.121	0.256	

Below diagonal: *p*-value in *Ar*, Above diagonal: *p*-value in UH_E



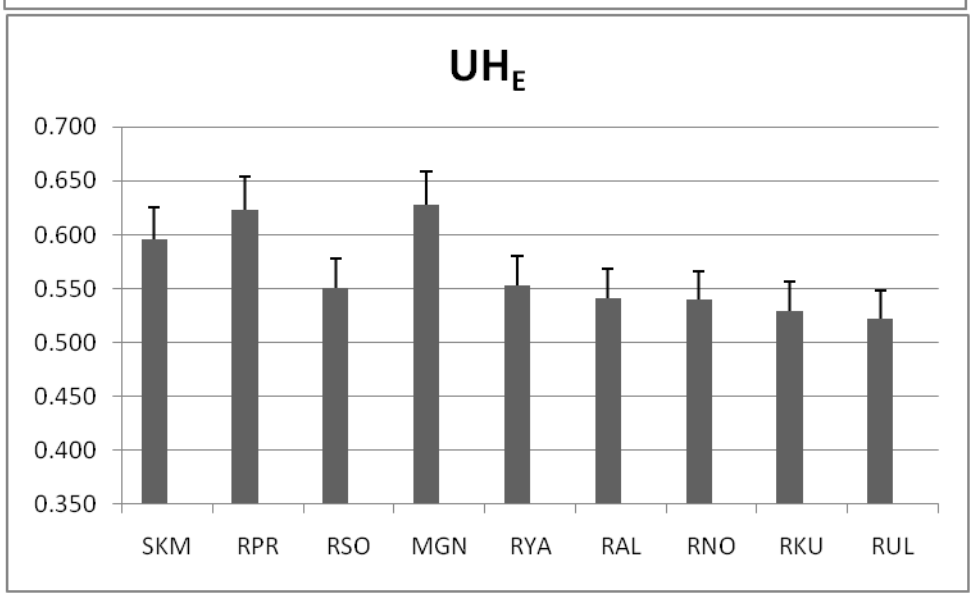


Figure 7. Bar graph of allelic diversity (*Ar*) and expected heterozygosity that are corrected by small sample sizes (UH_E) in eight Siberian roe deer population

Genetic relationship and gene flow

ENA-corrected (excluding null alleles) and uncorrected pairwise F_{ST} are shown in Table 10, where these two estimates did not show significant differences (Wilcoxon Rank Sum Test; U= 987, P= 0.8401). Therefore, we used uncorrected pairwise F_{ST} for further analyses and interpretation of genetic differentiation of Siberian roe deer population. Pairwise F_{ST} values for 24 out of 44 population pairs are significantly different from 0 after corrections for multiple comparisons ($P < 0.001$) (Table 10). The lowest value of genetic differentiation was detected in SKM vs. MGN (F_{ST}= 0.025) and roe deer from Jeju Island, South Korea (SKJ), showed the highest degree of genetic differentiation to all others (mean pairwise F_{ST}= 0.349). When a comparison is made between two regions (West vs. Central and East), roe deer in Urals and Kurgan, Russia (RUL and RKU) showed relatively higher degrees of genetic differentiation with Mainland Korea (SKM), Primorsky Krai, Russia (RPR) and Central Siberia (RSO and MGN) (mean pairwise F_{ST}= 0.122). The effective number of migrants per generation (N_em) ranged from 0.4 (SKJ vs. RYA, RSO, RAL, RNO, RUL and RKU) to 103 (RPR vs. MGN) (Table 10). Roe deer in Jeju Island, Korea (SKJ) showed negligible levels of gene flow relative to all others.

Table 10. Pairwise F_{ST} and gene flow (N_em in parentheses) estimates between geographic populations.

	SKJ	SKM	RPR	RYA	RSO	MGN	RAL	RNO	RUL	RKU
SKJ	—	0.277 (0.7)	0.279 (0.7)	0.366 (0.4)	0.355 (0.5)	0.295 (0.6)	0.376 (0.4)	0.372 (0.4)	0.393 (0.4)	0.387 (0.4)
SKM	0.286*(0.6)	—	0.011 (23.1)	0.072 (3.3)	0.030 (8.2)	0.029 (8.3)	0.092 (2.5)	0.095 (2.4)	0.138 (1.6)	0.387 (2.0)
RPR	0.290*(0.6)	0.009^{NS}(28.8)	—	0.046 (5.1)	0.007 (36.5)	0.011 (22.9)	0.065 (3.6)	0.081 (2.8)	0.115 (1.9)	0.095 (2.4)
RYA	0.373*(0.4)	0.068*(3.4)	0.044*(5.4)	—	0.038 (6.4)	0.056 (4.2)	0.054 (4.4)	0.045 (5.4)	0.054 (4.4)	0.055 (4.3)
RSO	0.366*(0.4)	0.020^{NS}(12.1)	−0.005^{NS}(inf)	0.041^{NS}(5.8)	—	0.006 (42.4)	0.070 (3.3)	0.091 (2.5)	0.134 (1.6)	0.099 (2.3)
MGN	0.299*(0.6)	0.025*(10.0)	0.002^{NS}(103)	0.051^{NS}(4.6)	0.000^{NS}(inf)	—	0.087 (2.6)	0.076 (3.0)	0.127 (1.7)	0.106 (2.1)
RAL	0.386*(0.4)	0.076^{NS}(3.0)	0.055^{NS}(4.3)	0.045^{NS}(5.3)	0.058^{NS}(4.1)	0.076^{NS}(3.0)	—	0.065 (3.6)	0.107 (2.1)	0.116 (1.9)
RNO	0.380*(0.4)	0.088*(2.6)	0.070*(3.3)	0.039^{NS}(6.2)	0.091^{NS}(2.5)	0.070*(3.3)	0.057^{NS}(4.2)	—	0.042 (5.8)	0.048 (5.0)
RUL	0.412*(0.4)	0.143*(1.5)	0.115*(1.9)	0.050*(4.8)	0.141*(1.5)	0.128*(1.7)	0.101^{NS}(2.2)	0.035^{NS}(7.0)	—	0.033 (7.4)
RKU	0.410*(0.4)	0.124*(1.8)	0.101*(2.2)	0.058*(4.1)	0.111*(2.0)	0.110*(2.0)	0.123^{NS}(1.8)	0.045^{NS}(5.3)	0.032^{NS}(7.6)	—

F_{ST} estimates (Weir and Cockerham, 1984) are below the diagonal and F_{ST} using the ENA correction are above the diagonal.
Probability of being different than zero after Bonfferoni correction for multiple comparisons (*$P < 0.001$, NS: not significant).

UPGMA trees based on Nei's D_A distances displayed topologies with three clusters (Figure 8). Relationship tree displayed Mainland Korea, Eastern and Central Siberia populations (SKM, RPR, RSO and MGN) clustered together with high bootstrap support (82%). However, the Jeju Island, South Korea (SKJ) population remains separated by long branches, possibly due to a founder effect.

Principal coordinates analysis (PCA) for all populations supported the result from the relationship tree, revealing similar patterns among locations (Figure 9A). PCA analysis performed without island population (SKJ) showed three clusters consisting of 1: Central and East (SKM, RPR, RSO and MGN), 2: West and Mid-west (RUL, RKU and RNO) and 3: Mid-west and Northeast (RAL and RYA) (Figure 9B).

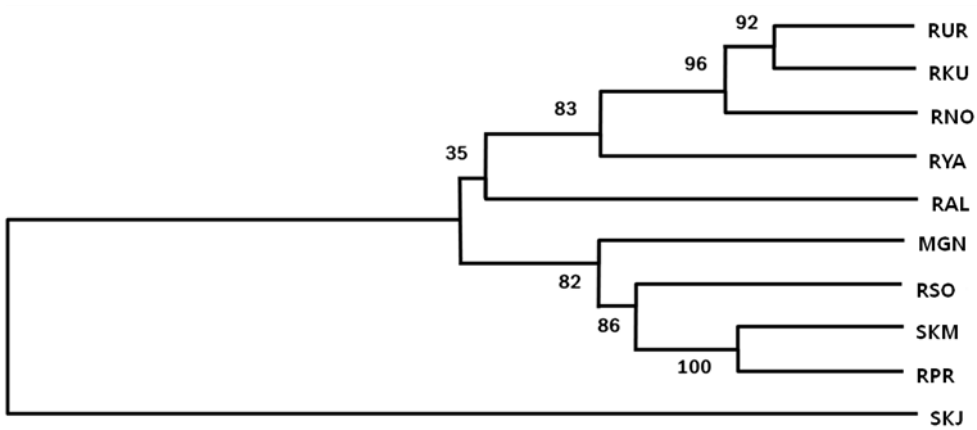
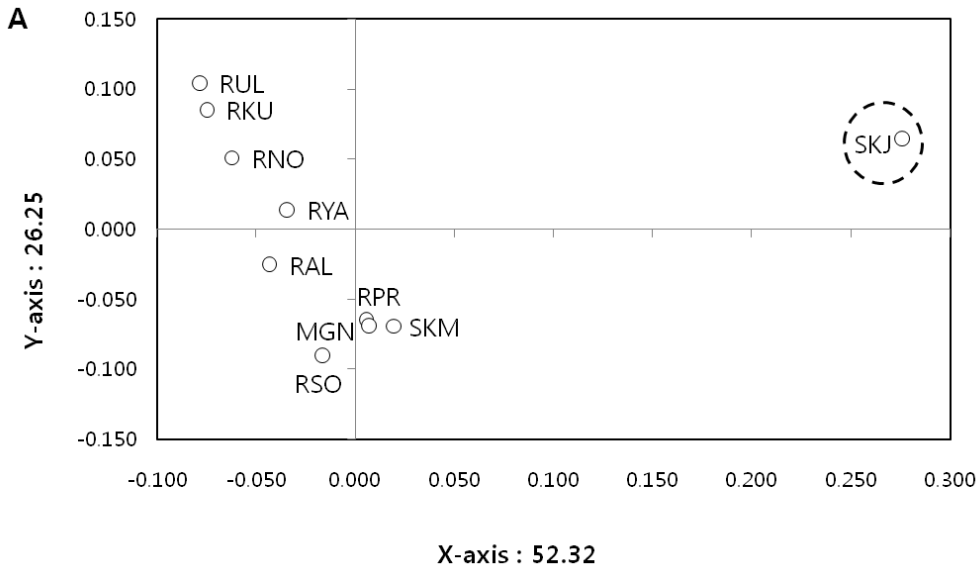


Figure 8. Relationship tree of Siberian roe deer from ten geographic locations. UPGMA tree was constructed based on Nei's D_A genetic distance.



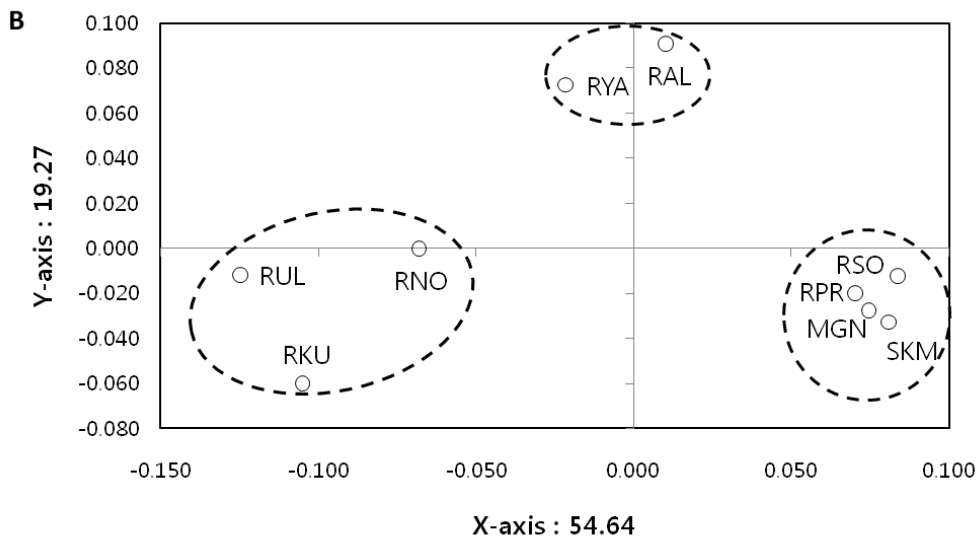


Figure 9. Scatter diagram of factor scores from a principal coordinate analysis of geographic locations.
A: Analysis for all populations, B: Analysis after excluding roe deer from Jeju Island. The percentage of total variation attributed to each axis is indicated.

Genetic structure

Bayesian model based clustering analysis identified three genetic clusters under the hierarchical island model suggested by the Evanno *et al.* (2005) (Figure 10). Initially, the highest K was observed when K was set to 2, dividing into Jeju Island, South Korea (SKJ) and all other locations. When Jeju Island, South Korea (SKJ), was excluded to detect sub-structuring in remaining cluster, two additional genetic clusters were observed, which clearly discriminated the population in Central and Eastern Siberia (SKM, RPR, RSO and MGN) from those in the Urals region and West Siberia, Russia (RUL, RKU and RNO) populations. Mountain Altay, Russia (RAL) and Yakutia, Russia (RYA) displayed intermediate genetic composition between the Central/Eastern and Western population. Overall, structure analysis under the hierarchical island model revealed three genetic clusters consisting of 1: Jeju Island, South Korea (SKJ), 2: Central and East (SKM, RPR, RSO and MGN; Southeastern group), and 3: West and Mid-west (RUL, RKU and RNO; Northwestern group) with admixed genetic compositions between the clusters 2 and 3 for Mid-west (RAL) and Northeastern (RYA) population. A pie chart represented for each sampling location on the map, apart from roe deer from Jeju Island, South Korea (SKJ), displayed two different genetic compositions with an admixed population observed in border areas (Figure 11).

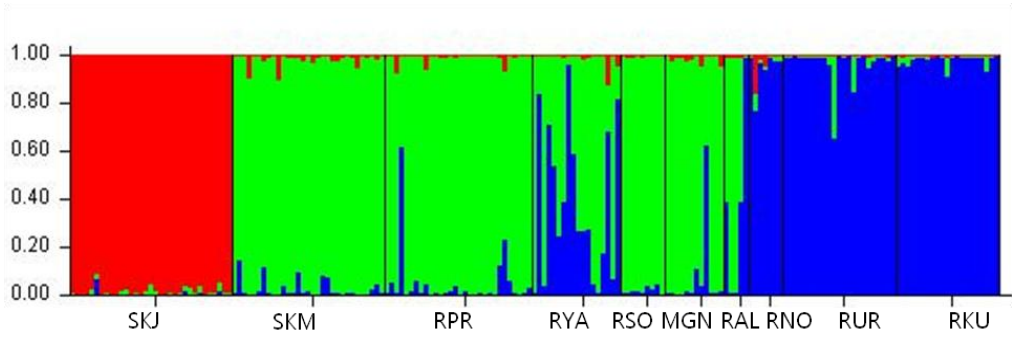


Figure 10. Bar plots for population structure estimates of Siberian roe deer. Population symbol on the x-axis indicates the putative population of sample origin. See Figure 11 for location abbreviation. Each color denotes a cluster from STRUCTURE analysis.

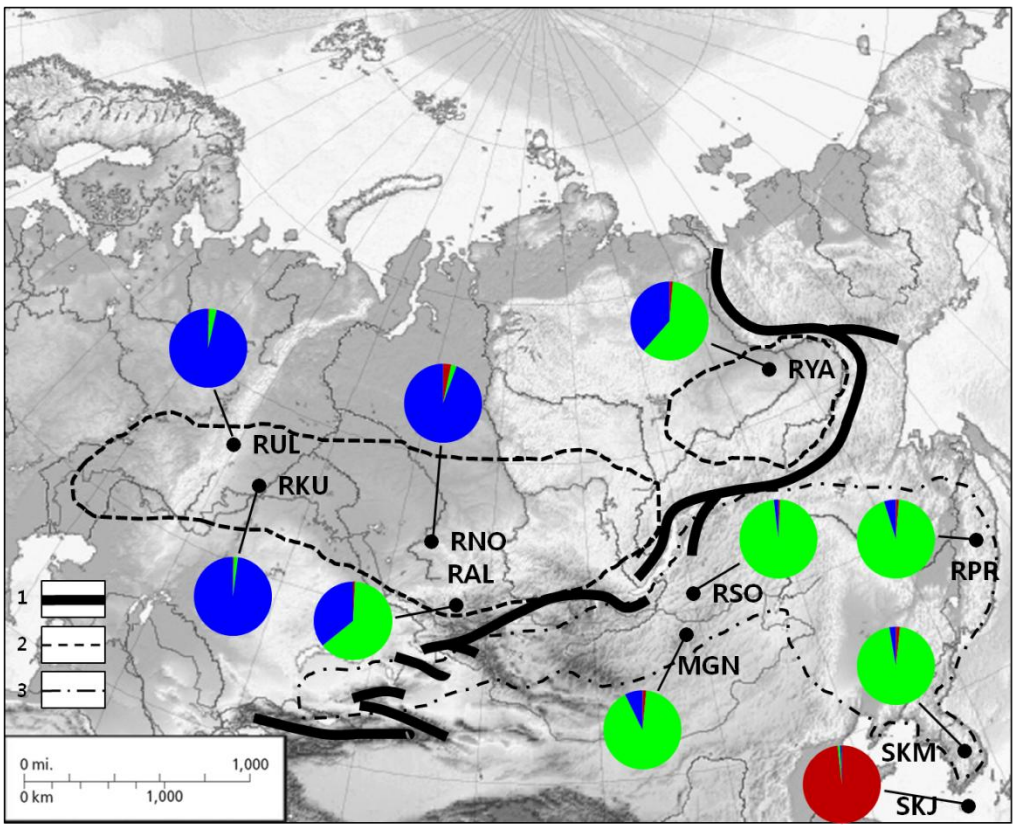


Figure 11. Sampling location and subspecies range of Siberian roe deer, *C. pygargus.* Pie charts of membership proportions of each sampled population inferred by structure analysis (K= 3).
1: Main Mountain ridges (Danilkin, 1996), 2: *C.p.pygargus*, 3: *C.p.tianschanicus,* SKJ: South Korea, Jeju (N= 33), SKM: South Korea Mainland (N= 31), RPR: Russia, Primorsky Krai (N= 30), RYA: Russia, Yakutia (N= 18), RSO: Russia, Sokhondinsky (N= 9), MGN: Mongolia, Northern part (N= 12), RAL: Russia, Altay (N= 5), RNO: Russia, Novosibirsk (N= 7), RUR: Russia, Ural (N= 23), RKU: Russia, Kurgan (N= 21).

Hierarchical analysis of molecular variance (AMOVA) analysis based on the geographical distance showed significant genetic differentiation (F_{RT}= 0.148) among regions, which was much higher than among population within regions (F_{SR}= 0.040) (Table 11A). Result based on the three clusters after two admixed regions (RYA and RAL) excluded presented greater difference in genetic differentiation among regions (F_{RT}= 0.200) (Table 11B), supporting the obvious genetic differentiation among three clusters; Jeju Island, Korea (SKJ), Eastern region (SKM, RPR, MGN and RSO) and Western region (RNO, RUL and RKU). In addition, AMOVA analysis based on the two clusters after Jeju and two admixed regions (RYA and RAL) excluded showed genetic differentiation among regions (F_{RT}= 0.093) and among population within regions (F_{SR}= 0.020) (Table 11C).

Table 11. Analysis of molecular variance (AMOVA) of the Siberian roe deer populations based on various geographic/genetic groupings (four geographic regions, three genetic clusters, and two geographic regions).

A								
Source of variation	df	SS	MS	Est. Var.	%	*F*-Statistics	Value	P-Value
Among regions	3	203.555	67.852	0.615	15	F_{RT}	0.148	0.001
Among pop[a]	6	50.962	8.494	0.142	3	F_{SR}	0.040	0.001
Among individuals	179	733.874	4.100	0.710	17	F_{ST}	0.182	0.001
Within individuals	189	506.500	2.680	2.680	65	F_{IS}	0.209	0.001
Total	377	1494.892		4.147	100	F_{IT}	0.354	0.001
B								
Source of variation	df	SS	MS	Est. Var.	%	*F*-Statistics	Value	P-Value
Among regions	2	192.296	96.148	0.853	20	F_{RT}	0.200	0.001
Among pop[a]	5	33.272	6.654	0.077	2	F_{SR}	0.022	0.001
Among individuals	158	627.752	3.973	0.640	15	F_{ST}	0.218	0.001
Within individuals	166	447.000	2.693	2.693	63	F_{IS}	0.192	0.001
Total	331	1300.319		4.263	100	F_{IT}	0.368	0.001
C								
Source of variation	df	SS	MS	Est. Var.	%	*F*-Statistics	Value	P-Value
Among regions	1	53.813	53.813	0.370	9	F_{RT}	0.093	0.001
Among pop[a]	5	33.272	6.654	0.071	2	F_{SR}	0.020	0.001
Among individuals	126	524.919	4.166	0.645	16	F_{ST}	0.111	0.001
Within individuals	133	382.500	2.876	2.876	73	F_{IS}	0.183	0.001
Total	265	994.504		3.962	100	F_{IT}	0.274	0.001

A: Four regions: Jeju Island (SKJ), East region (SKM, RPR), Central region (RYA, RSO, MGN) and West region (RAL, RNO, RUL, RKU). B: Three genetic clusters with two admixed populations (RYA and RAL) excluded: Jeju Island (SKJ), Eastern region (SKM, RPR, RSO, MGN) and Western region (RNO, RUL, RKU). C: Two geographic regions with SKJ and two admixed populations (RYA and RAL) excluded: Eastern region (SKM, RPR, RSO, MGN) and Western region (RNO, RUL, RKU). df: degrees of freedom; SS: sum of squares; MS: mean squares; Est. Var.: estimated variance within and among populations; [a] Among population within regions.

The Barrier analysis based on the pairwise F_{ST} verified three areas of relatively sharp change in genetic composition (Figure 12). The first barrier separated the Eastern region (SKM, RPR, MGN and RSO) from West and Mid-west region (RAL, RNO, RUL and RKU) with supported by six to eleven loci. The second barrier separated Northeastern population (RYA) from all other populations with supported by three to eleven loci. The third barrier, supported by two to eleven loci, separated Mid-west population (RAL) from Western region (RNO, RUL and RKU).

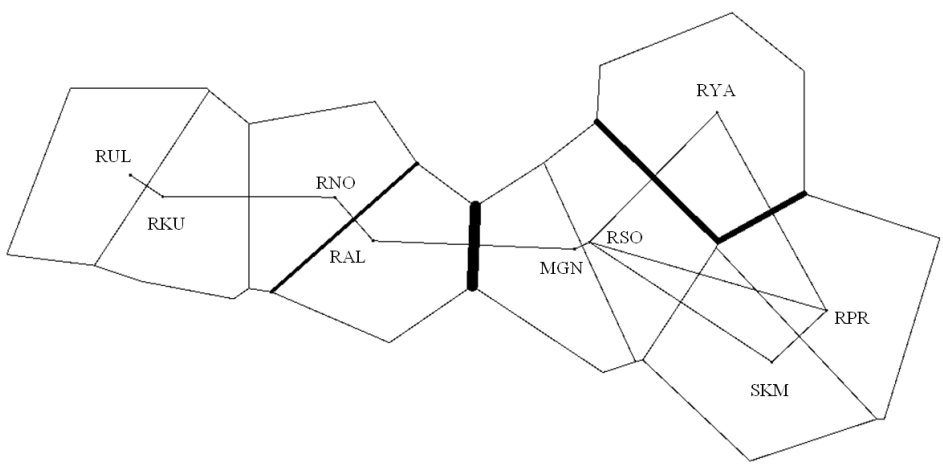


Figure 12. Areas of limited gene flow as estimated by BARRIER using Monmorier algorithm (Manni *et al.*, 2004). The genetic barriers are shown in bold lines, which are proportional to the intensity of the barriers.

Regression of the genetic isolation by geographic distance (IBD) over all samples showed significant correlation in both with and without Jeju Island included (Figure 13). However, relationship between genetic and geographic distances was increased as high as 3.5 fold when Jeju Island, Korea (SKJ), was removed, indicating that the distinct genetic differentiation of SKJ from other populations greatly decreased the IBD relationship. Also, IBD with marked pair of each population based on the two clusters (structure) showed slightly deviated point from standard linear which typically distributed on the low (pair of population within cluster) and high (pair of population between clusters) genetic distance (Figure 13B).

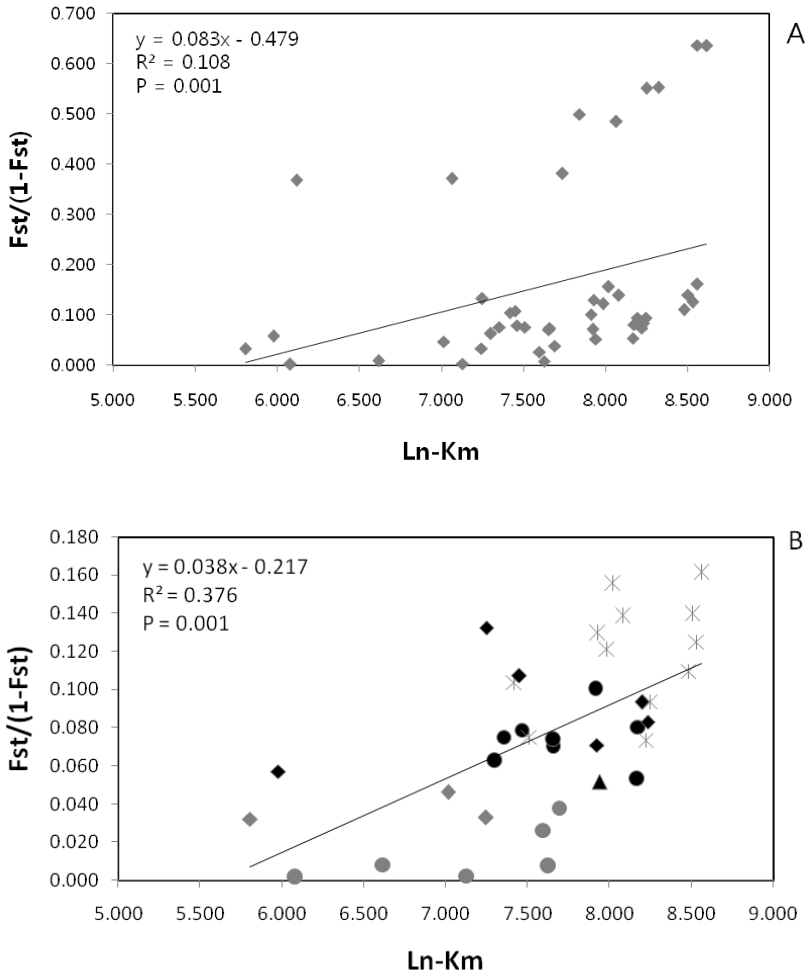


Figure 13. Regression of genetic distance on geographic distance between pairs of geographic Siberian roe deer populations.
A: Analysis for all populations, B: Analysis after excluding roe deer from Jeju Island. Each diagram and color present pairs of population based on the structure result (two clusters). Mantel's test for correlations was carried out with 999 permutations.
●: within East cluster (SKM, RPR, MGN and RSO), ◆: within West cluster (RNO, RUL and RKU), ●: between mixed populations (RAL and RYA) and East cluster, ◆: between mixed populations (RAL and RYA) and West cluster, ▲: within mixed populations (RAL and RYA), ✳: Between East and West cluster (opposite side of the mountains)

To provide insights into the main causes of these three regions (SKJ, Eastern region and Western region) differentiation, statistical comparing p$R_{ST,}$ F_{ST} and R_{ST} values (drift vs mutation) were performed. pR_{ST} values were very similar to F_{ST} and permutation tests did not detect R_{ST} value significantly higher ($p < 0.05$) than pR_{ST} except one locus RT30 (Table 12). This suggests that differentiation is caused mainly by drift. This result also ascertains the restricted level of gene flow between populations separated by the high mountain ridges and implies that F_{ST} should be a better estimator than R_{ST} of population differentiation for Siberian roe deer.

Table 12. Differentiation among three regions (cluster) of Siberian roe deer estimated by pairwise R_{ST}, mean pR_{ST} and F_{ST} values per locus and multilocus.

Locus	NA	R_{ST}	pR_{ST} (C.I.) [b]	F_{ST}
Multilocus	10.17	0.171 NS [a]	0.119 (0.055−0.196)	0.124
RT1	18	0.177 NS	0.128 (0.007−0.246)	0.167
RT20	6	0.111 NS	0.086 (−0.012−0.162)	0.101
RT23	5	0.107 NS	0.081 (0.030−0.133)	0.094
RT24	6	0.101 NS	0.107 (0.016−0.186)	0.123
RT30	22	0.308*	0.140 (0.038−0.276)	0.168
Roe01	5	0.048 NS	0.074 (0.032−0.090)	0.079
Roe09	4	0.009 NS	0.031 (0.008−0.048)	0.033
MB25	2	0.201 NS	0.201 (0.201−0.201)	0.201
BM757	24	−0.012 NS	0.053 (−0.018−0.160)	0.063
CSSM41	10	0.021 NS	0.107 (0.019−0.183)	0.128
IDVGA8	16	0.068 NS	0.117 (−0.007−0.305)	0.140
IDVGA29	4	−0.016 NS	−0.023 (−0.034−−0.013)	−0.021

[a] probability values of allele size permutation tests on R_{ST} (*P < 0.01, NS: not significant). [b] 95% confidence interval (C.I.) is obtained after 1000 random permutation of the allele size. NA: Number of alleles

Three different measures of detecting population genetic bottlenecks revealed no evidence of a historical or recent bottleneck for nine populations (SKM, RPR, RYA, RSO, MGN, RAL, RNO, RUL and RKU) (Table 13). However, the event of a recent population bottleneck was detected in the Jeju Island, South Korea (SKJ) (Wilcoxon sign-rank test, two-phase mutation model (TPM) = 0.005), implying significant excess of heterozygosity relative to drift-mutation equilibrium. At the same time the Garza and Williamson's (2001) M values (0.765) and mode shift (none) tests did not show any evidence of genetic bottleneck. Bottleneck analysis suggested that all populations, except Jeju Island, South Korea (SKJ), were in the range of a historically stable population.

Table 13. Results of various tests to detect a recent population bottleneck event within geographic populations.

Pop	Wilcoxon sign-rank tests[a]	Mode shift	M^b
	TPM		
SKJ	0.005	None	0.765 (0.040)
SKM	0.266	None	0.885 (0.009)
RPR	0.519	None	0.929 (0.018)
RYA	0.380	None	0.777 (0.058)
RSMG	0.733	None	0.831 (0.037)
RSO	0.831	None	0.793 (0.052)
MGN	0.850	None	0.753 (0.048)
RARN	0.320	None	0.810 (0.057)
RAL	0.365	Shifted mode	0.769 (0.103)
RNO	0.206	Shifted mode	0.840 (0.055)
RURK	0.969	None	0.820 (0.058)
RUL	0.677	None	0.787 (0.073)
RKU	0.151	None	0.826 (0.069)

[a] One-tail probability for observed heterozygosity excess relative to the expected equilibrium heterozygosity (H_{eq}), which is computed from the observed no. of alleles under drift-mutation equilibrium. TPM, two-phase model.

[b] M value and its variance (in parentheses) of Garza and Williamson. M= the mean ratio of the no. of alleles to the range of allele size.

Discussion

In this study, we investigated the variability of microsatellite loci to understand how different factors of genetic diversification such as isolation by distance, isolation by geographical barriers could affect the genetic diversity and population structure of Siberian roe deer in Northern Asia. Our study is based on samples from extensive geographic areas of Northern Asia, from Ural Mountains to the Korean Peninsula and Jeju Island, covering most of the species' range to clarify the genetic relationships among populations from different geographical locations. Autosomal nuclear markers of microsatellites were employed to investigate the levels of genetic variation and genetic structuring of Siberian roe deer populations.

Genetic diversity of Siberian roe deer

Relative comparison of genetic diversity estimates among other roe deer species/populations would be informative to understanding of the present genetic status of Siberian roe deer. Although different sets of microsatellite loci were employed, apart from populations in Jeju Island, South Korea (SKJ), most of Siberian roe deer populations revealed moderate levels of genetic diversity (H_E= 0.522−0.628), compared to those previously reported for European roe deer. Microsatellite diversity of European roe deer ranged from 0.17 to 0.79 in several locations from Italy, Britain and northern Germany (H_E= 0.17−0.58, H_E= 0.59−0.62 and H_E= 0.74−0.79, respectively) (Lorenzini *et al.*, 2002; Zachos *et al.*, 2006; Baker and

Hoelzel, 2013). However, because the different sets of microsatellites were employed in diversity estimates and this may cause an inherent ascertainment bias that can vary among primer pairs, especially in different species, it should be interpreted with caution.

During the 20th century, many of the local Siberian roe deer populations were significantly abated as a result of human interference (Ushkov, 1954; Filonov, 1974; Shvets, 1975; Kucherenko and Shvets, 1977; Danilkin and Dulamtseren, 1981). However, present data on the genetic diversity of Siberian roe deer suggests that the historical population reduction was transient, and its effects on the genetic diversity of the populations were insignificant. Result of bottleneck test also supported the lack of evidence for bottleneck event, except in the Jeju Island population (See below), indicating general stability of Siberian roe deer populations in continental Asia.

Different measures of microsatellite variability are consistently high in populations from East and Central Asia compared to West Siberia (Table 8 and figure 7). One reasonable assumption is that areas to the south and east of Siberia have function as refugia for roe deer during glacial periods. Several vertebrate species were also reported to have high levels of mitochondrial DNA variations in eastern Russia compared with those of surrounding areas (Kryukov, 2010). Combination of cold open steppes with forested areas in south and east of Siberia may have resulted in highly diverse faunas (Zabelin, 2012), which could provide preservation and diversification of genetic lineages. However, phylogeographic and archaeological

inference with additional samples from different geographical regions should be implemented to precisely determine the role of this region as refugia.

Roe deer from Jeju Island, South Korea (SKJ) showed the lowest level of genetic diversity among Siberian roe deer that were sampled in this study. This presumably is due to the geographic isolation and historical population fluctuations on Jeju Island. Roe deer inhabited in Jeju Island during the last glacial maximum (LGM) when there was a bridge between the island and the Korean peninsula. It is probable that a relatively small group of animals was founded in the island after the last glacial periods, which led to reduced genetic diversity due to processes such as founder effect and genetic drift. Human interference, such as excessive hunting and poaching, could be another possible cause of the genetic deprivation in Jeju population. The roe deer population in Jeju gradually declined to near extinction in the early 1970s because of continuous hunting and poaching (Choi, 2011). Since the 1980s, Jeju Special Self-Governing Province and Jeju citizens has been active in conservation for roe deer such as providing food during winter, removing traps, and clamping down on poaching (Yoon, 2003; Oh, 2004). Consequently, the roe deer population in Jeju increased to 5,000 individuals in 1992 and climbed to 12,881 individuals in 2009 (Choi, 2011). The effect of recent fluctuations of roe deer population in Jeju Island on its genetic diversity is supported by the Bottleneck tests (Table 13). Therefore, continuous monitoring of genetic diversity would be essential for

effective management and conservation of Siberian roe deer in Jeju Island.

Genetic structure and gene flow

Present studies of genetic structure and differentiation among Siberian roe deer populations clearly display the existence of genetically distinct three clusters which comprise of the southeastern group (SKM, RPR, RSO and MGN), northwestern group (RUL, RKU and RNO) and Jeju Island population in Korea (SKJ). Such pattern of genetic structure is well in accordance with distribution of the two subspecies, *C. p. pygargus* and *C. p. tianschanicus*, suggested by previous study (Danilkin, 1999). Recently, mitochondrial DNA sequence and nuclear IRBP (Interphotoreceptor retinoid binding protein) data has been presented that Jeju Island population to another subspecies, *C. p. ochracea* (Koh *et al.*, 2013). The genetic makeups of the two populations (RYA and RAL) are indicative of admixture of the two groups (southeastern and northwestern groups); however, a small sample size limits ultimate defining of their genetic status.

A previous study (Danilkin, 1996) proposed three major factors that may limit the geographical distribution of Siberian roe deer. The first factor is geographical barriers consisting of major mountain ridges (Altai, Sayans and Stanovoye) and the Lake Baikal (Figure 11), which also delineate geographical ranges of two subspecies (*C. p. pygargus* and *C. p. tianschanicus*). The second factor is the depth of snow and duration of the snowy period

(Formozov, 1946; Nasimovish, 1955; Danilkin, 1996) and last factor is the predominant vegetation type of the region, such as taiga, tundra, and desert (Danilkin, 1996). These three factors and their interaction presumably limited further spread of roe deer, but probably first factor is the most important for the formation of genetic groups or subspecies. Besides, according to the data of annual depth of snow (Appendix S2) and duration of the snowy period (Appendix S3), increase in the duration of the snowy period is conformed to major mountain ridges and genetic structure. Although the data in the whole area of Russia cannot accurately reflect the height and environment of major mountain ridges, probably the duration of the snowy period had more influence on the formation of genetic group than depth of snow. The other possible reason of it is that the mountain ridges could serve as refugia during periods of climate change (e. g. during the glacial maximums). In the periods of climatic optimums different genetic lineages could spread from the mountains in different areas resulting in formation of genetically different groups, possibly subspecies.

Barrier analysis that detected change genetic composition was also support limited gene flow in the major mountain ridges (Figure 12). Southeastern group (SKM, RPR, RSO and MGN) and Northwestern group (RUL, RKU and RNO) supported relatively high frequency and fallowed by genetically admixed two populations (RYA and RAL) in the border areas. Besides, results of the Isolation by distance (IBD) (Figure 13B) displayed that about 38%

of the genetic variation is explained by geographical distances between locations over the entire continent of Asia, which fits the hierarchical island model, suggesting modern genetic structure resulted from natural processes (Danilkin, 1992; Danilkin *et al.*, 1992; Danilkin, 1995; Danilkin, 1996). Additionally, different pattern of distribution in the IBD scatter plot between and within groups (southeastern and northwestern groups) ascertains the effect of mountains ridges on the restricted level of gene flow between groups. Thus, mountain ridges of the southern Siberia have limited gene flow between Southeastern (SKM, RPR, RSO and MGN) and Northwestern (RUL, RKU and RNO) groups, leading to current genetic structure.

It should be noted that the Altay population (RAL) is located in the border area of two subspecies and shows the admixed pattern of two genetic clusters. This population is genetically related to both groups (Southeastern and Northwestern) and likely has historical and ongoing gene flow with adjacent locations (Figure 11). A previous study of mitochondrial DNA (Vorobieva *et al.*, 2011) proposed that roe deer in Altai Mountain might experience multiple population replacements, stressing the role of the Altai Mountain as a physical boundary separating *C. p. pygargus* and *C. p. tianschaniscus*. This speculation is based on the genetic heterogeneity of Siberian roe deer in the Altai Mountains, and relatively stable climatic conditions of the region compared to other Siberian regions during the Pleistocene (Vorobieva *et al.*, 2011). However, to resolve the question of border area, additional

population genetic studies with more samples from areas at a finer geographic scale will be required.

Roe deer population in Yakutia, Russia (RYA), were established as a result of natural radiation from the southern parts of geographical range and could originate from both *C. p. pygargus* and *C. p. tianschaniscus* (Argunov, 2013). This assumption complies with the genetic structure of the Yakutian population obtained in this study and is also confirmed by the previous studies using morphology and karyotype (Boeskorov and Danilkin, 1998).

Roe deer from Jeju Island, South Korea (SKJ) are genetically divergent from all other Siberian roe deer, including those on the Korean mainland. The Jeju Island population was isolated from the mainland population since LGM, and as a result, there has been no gene flow between these two locations. Thus, the present genetic feature of the Jeju Island population was derived as a consequence of long-term geographical isolation and adaptation to island environment. Cases where Jeju island populations showing unique genetic and/or morphological features was also described for other mammal species such as wild boar (*Sus scrofa*), striped field mouse (*Apodemus agrarius chejuensis*) and Siberian weasel (*Mustela sibirica*) (Jo *et al.*, 2012). Future studies of this isolated population would contribute to understanding the effect of peripheral isolation on microevolution in Cervidae.

Our results do not coincide with the recent phylogeographic findings (Lorenzini *et al.*, 2014) that demonstrated no apparent geographical structuring for Siberian roe deer sampled from vast

geographic areas of Eurasia. Variability of mtDNA control region suggested that the Siberian roe deer in Asia has undergone genetic admixture and appears to show no apparent geographic barriers to gene flow (Lorenzini *et al.*, 2014). This difference could be due to the sensitivity of molecular markers and disparate interpretation owing to insufficient sample size and different modes of inheritance. The microsatellites are highly polymorphic and autosomal nuclear markers with biparental inheritance, and are more appropriate to delineate genetic structure of recently diverged populations.

Management and Conservation Implications

Overall, this study suggests that at least three distinct management units may exist for the Siberian roe deer populations in Asia (Palsboll *et al.*, 2007): Northwest genetic group (RUL, RKU and RNO, partially corresponding to *C. p. pygargus* subspecies), southeast genetic group (SKM, RPR, RSO and MGN, corresponding to *C. p. tianschanicus*) and Jeju Island genetic group. Future planning of management and/or conservation policies, including *ex situ* population breeding, translocation and reintroduction programs, need to consider the distinctiveness of the three genetic groups in the Siberian roe deer species. Strict application of management unit concept for the two admixed populations (RYA and RAL) might be relaxed, or postponed until more detailed studies focusing on these populations are performed.

The roe deer population in Jeju Island, Korea (SKJ) needs special attention due to its low level of genetic diversity compared

to those of continental populations. The Jeju Island population seems to be thriving at the present time, despite the low level of heterozygosity. The current size of the Jeju roe deer population is estimated to be around 12,881 (Choi, 2011) and considered to be over-populated in the island. However, considering the deprived level of genetic diversity, it is probable that the Jeju population might be vulnerable to epidemic diseases or any change of environment in the future. Therefore, it is recommended that both the genetic and physical health statuses of the population are closely monitored. Artificial translocation of roe deer individuals from the mainland Korea to Jeju Island to increase genetic diversity of Jeju population is not recommended because these two populations are genetically highly differentiated and should be regarded as separate management units.

Herbivorous animals such as roe deer play an important role in the ecosystem, providing a prey for large carnivores. Therefore, proper genetic management of Siberian roe deer populations and continuous monitoring of its genetic status is critical for maintaining healthy ecosystem. It is important to stress that systematic cooperation between countries where Siberian roe deer inhabit (Russia, Kazakhstan, Mongolia, China, North Korea and South Korea) is imperative for effective maintenance of genetic diversity and gene flow of Siberian roe deer. In particular, cooperative management of border area is important not only for the roe deer itself but also for a number of endangered large carnivore species. For example, Siberian roe deer is one of the main prey animal of

Amur leopard (*Panthera pardus orientalis*) in the border area among Russia, China and North Korea (Heptner *et al.*, 1992; Hebblewhite *et al.*, 2011). Thus maintaining healthy roe deer population in this transboundary region is crucial for the survival of Amur leopard, which is one of the most severely endangered subspecies of large Felidae species in the world (Heptner *et al.*, 1992; Miquelle *et al.*, 1999; Peterson and Ciucci, 2003; Molinari-Jobin *et al.*, 2007; Hebblewhite *et al.*, 2011). The status of the Siberian roe deer population in North Korea remains unknown and the gene flow has been discontinued along the Demilitarized Zone (DMZ) of North and South Korean border for more than five decades. This situation would have negative impacts on the long-term persistence of the Siberian roe deer in Korean peninsula and the restoration efforts of Amur leopard and tiger populations in this region. Siberian roe deer also serve as an important prey species for other carnivores like Amur tigers, gray wolves, lynxes, dholes, bears, as well as foxes, martens, eagles and wild boars (Geist, 1998; Miquelle *et al.*, 1999). Thus, proper management of roe deer populations in northern Asian continent will also benefit many other species, and eventually, the biodiversity of the entire region.

CHAPTER Ⅲ.

Phylogeography of Siberian musk deer (*Moschus moschiferus*) in South Korea based on mitochondrial DNA

Introduction

The Siberian musk deer (*Moschus moschiferus*) is one of the most widespread species of the genus *Moschus* in the family Moschidae. Siberian musk deer is forest animal inhabited in mixed coniferous and broadleaf forest in mountainous regions. It is distributed widely in the Russian Federation (Siberia and the Far East), eastern Kazakhstan, northeastern and northwestern China, Mongolia and Korea (Tsendjav, 2002; Baskin and Danell, 2003; Nyambayar *et al.*, 2015). Musk deer, on the basis of morphological and genetic study, have been classified into different species from only one species (Sokolov and Prikhod'ko, 1997), through 5 species (Groves *et al.*, 1995; Su *et al.*, 1999), and 6 species (Li *et al.*, 1999; Su *et al.*, 2001) to 7 species (Groves and Grubb, 2011; Pan *et al.*, 2015). Nonetheless, Siberian musk deer has been a typical species and has been always classified as a species. Recently, most authors agree that Musk deer consists of 7 subspecies. However, the subspecies classification of Siberian musk deer is controversial and studies are in the initial stage in the world.

Siberian musk deer (*M. moschiferus*) was classified three subspecies: *M. m. moschiferus* (Siberia, Mongolia, Northwest Heilongjiang), *M. m. parvipes* (Russian Far East, Korea, South Heilongjing) and *M. m. sachalinensis* (Sakhalin) based on characteristics of external and skull morphology (Groves *et al.*, 1995; Groves and Grubb, 2011). While, Sokolov and Prikhod'ko (1997, 1998) suggested that there are five subspecies with the color features, skull and pelage: *M. m. moschiferus* (Siberia and mongolia), *M. m. turovi* (Russian Far East), *M. m. arcticus* (Verkhoyansk Ridge), *M. m. parvipes* (Korea) and *M. m. sachalinensis* (Sakhalin). Recently, many authors have followed the subspecies classification system of Sokolov and Prikhod'ko (1997, 1998). However, the morphological and ecological studies on Siberian musk deer subspecies have not been published since the 2000s.

Molecular genetic studies mostly focus on establishing the relationship between species of musk deer, evolutionary history, and genome structure, until now (Su *et al.*, 1999; Li *et al.*, 1999; Su *et al.*, 2001; Jang and Hwang, 2010; Pan *et al.*, 2015; Yang *et al.*, 2015). There is one single study on the relationship between Siberian musk deer subspecies and their genetic diversity using mtDNA control region (Kholodova and Prikhodko, 2006). This study demonstrated that musk deer from the Russian Far East (*M. m. turovi*) and Sakhalin Island (*M. m. sachalinensis*) were genetically similar with one phylogroup but Sakhalin musk deer form a distinct cluster within this phylogroup. Also, the distribution of musk deer

occurred from Eastern Siberia to the Sakhalin Island through Russian Far East. However, the study did not include Verkhoyansk and Korean subspecies (*M. m. arcticus* and *M. m. parvipes*).

Internationally, Siberian musk deer is classified as Vulnerable (VU) by the IUCN and CITES Appendix Ⅱ (Nyambayar *et al.*, 2015). One large reason that musk deer are endangered is the loss of habitat and overhunting by human (Wemmer, 1998; Homes, 2004). The odor musk that male musk deer have is very strong. So, the musk has been used as not only materials of perfume but also expensive oriental medicine since a long time ago. Therefore, in order to obtain musk, a lot of illegal poaching has been performed. In South Korea, Siberian musk deer are estimated to have lived along the Mt. Taebaek before and locally abundant in the high mountainous regions. However, the distribution of Korean subspecies (*M. m. parvipes*) had a extremely decreased from 1950s to 1999 (Lee and Rhim, 2002). Up to now, South Korea designated them as the natural monument and class Ⅰ endnagered species (Won, 1992). Therefore, following the examples of Mongolia and China, South Korea also started its efforts to restore of musk deer.

It is important to check the genetic status of population as well as carry out an ecological study of habitat for the successful and accurate restoration and re-introduction of endangered species (Kim *et al.*, 2011). Genetic analysis can provided taxonomical information that can support the carrying adequate restoration programs (Lee *et al.*, 2008). Genetic variation of population or species is also considered important for restoring threatened animal

and conservation genetics (Avise, 2004). The restoration of Asiatic black bear (*Ursus thibetanus*) in South Korea is a great example. Phylogenetic and population analysis of Asiatic black bear using mitochondrial and microsatellite markers helped to identify the properly conservation units based on evolutionary significant units (Hong, 2005; Kim *et al.*, 2011). However, there have been few studies on Korean musk deer habitats (Kim *et al.*, 2007; Park *et al.*, 2008) and no study has been released on molecular marker-based genetic diversity and phylogenetic relationship for musk deer in korea.

Overall, phylogeography studies using mtDNA sequences of the Siberian musk deer can provide basal information to better understand the present genetic status of Korean subspecies (*M. m. parvipes*). This study investigates the genetic relationship of the Korean subspecies with other subspecies and the extent of genetic diversity. The insights obtained from this study can be applied in the future reintroduction and conservation of Siberian musk deer in South Korea.

Materials and Methods

Sample collection and DNA extraction

A total of 13 Siberian musk deer (*M. moschiferus*) hair and DNA samples were collected from three location and different subspecies in Russian Far East (*M. m. turovi*, n= 8), Northeastern China (*M. m. moschiferus*, n= 1)and South Korea (*M. m. parvipes*, n= 4)(Figure 14 and Appendix S4). This experimental work was conducted with permission by the Conservation Genome Resource Bank for Korean Wildlife (CGRB) that provided the musk deer samples for this study. All samples were legally collected and deposited into CGRB. The procedures involving animal samples followed the guidelines by Seoul National University Institutional Animal Care and Use Committee (SNU IACUC). Collected samples were frozen at −70 ℃ deep freezer in the CGRB until DNA extraction. Genomic DNA was extracted from individual sample using the DNeasy tissue and blood kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

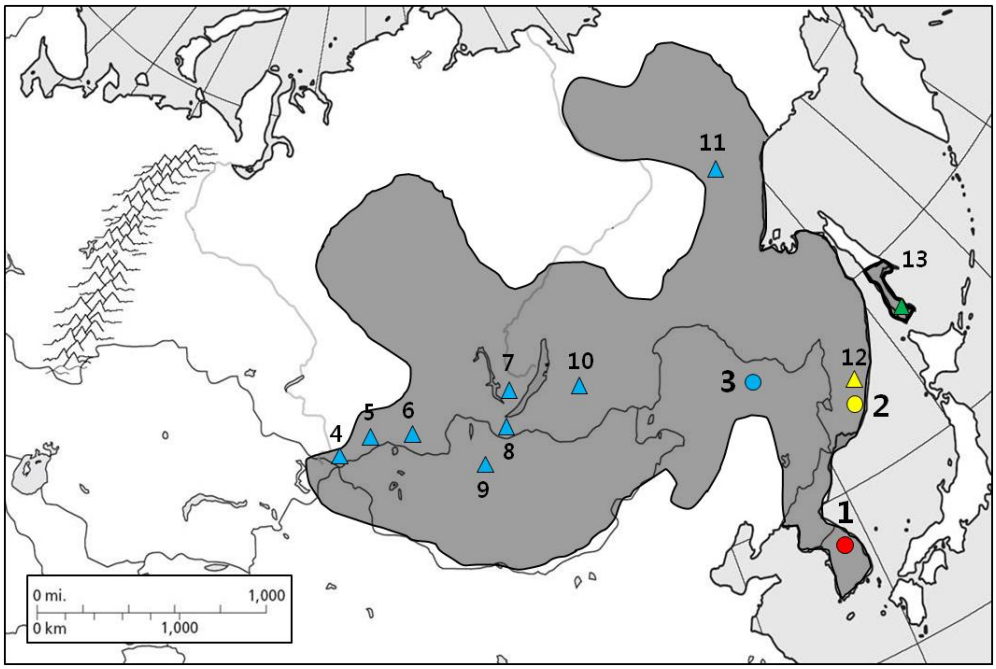


Figure 14. Distribution of Siberian musk deer (*Moschus moschiferus*) and study locations. The number and diagram indicate sampling locations; 1= Hwacheon, Gangwon-do, South Korea, 2= Lazo district, Primorsky Krai, Russia, 3= Heilong Jiang, China. The Sampling locations from in this study designated "circle" and locations of published data (Kholodova and Prikhodko, 2006; Jang and Hwang, 2010) from NCBI designated "triangle". The color indicates each subspecies followed the classification system of Sokolov and Prikhod'ko (1997, 1998): Red= *M. m. parvipes*, Yellow= *M. m. turovi*, Green= *M. m. sachalinensis,* and Blue= *M. m. moschiferus*. See Appendix S4 for specific sampling site information of number 4~13.

PCR amplification and DNA sequencing

Genomic DNA was extracted from hair root using the Gentra Puregene tissue kit (Qiagen, USA). The hypervariable fragment of mtDNA control region (left domain) was amplified by polymerase chain reaction (PCR) using primer Pro (L15376): 5' – CAC TAT CAA CAC CCA AAG CTG AAG – 3' and Dlc (H16498): 5' – ATG GCC CTG AAG AAA GAA CCA GAT G – 3' (Kholodova *et al.*, 2001). The PCR reaction conditions were: 94℃ for 5 min; 35cycles of 94℃ for 1 min, 47℃ for 1 min, and 72℃ for 2 min; and finally 72℃ for 10 min. The amplification was carried out in 20 ㎕ reaction volume containing 10 – 100 ng template DNA, 100 μM each dNTPs, 10 pmole each primer, 1.5 mM $MgCl_2$, 1 unit i-star Taq™ DNA polymerase (iNtRON Biotechnology Inc, Korea), and 1 x PCR buffer. The PCR products were purified with Zymoclean™ Gel DNA Recovery Kit (ZYMO RESEARCH, USA). Purified PCR products were sequenced using ABI Prism™ 377 automated sequencer (Applied Biosystems Inc, USA). The sequencing primers for mtDNA control regions were the same as those used for the amplification.

Data analysis

The sequences obtained in this study were identified as *Moschus* species through BLAST searches (Altschul *et al.*, 1997) and aligned with ClustalX version 1.83 (Thompson *et al.*, 1997). To obtain a comprehensive genetic relationship between subspecies, 35 (individual sequence) or 25 (haplotype) published mtDNA control

region (300bp) from NCBI were used to analysis (Appendix S4). Haplotype diversity (Hd), and nucleotide diversity (π) for each regions (subspecies) were estimated with DNASP version 5.1 (Librado and Rozas, 2009). Pairwise genetic distance between populations calculated using MEGA 5.05 (Tamura *et al.*, 2011) with Kimura's two parametric model (Kimura, 1980). The ARLEQUIN 3.1 (Excoffier *et al.*, 2005) was used to calculate pairwise genetic differentiation among geographical regions.

Phylogenetic trees to identify evolutionary relationships were constructed using three methods: Neighbor-joining (NJ: Saitou and Nei, 1987) using Kimura's two parametric model (Kimura, 1980), Maximum parsimony (MP) and Maximum-likelihood (ML). We used the fragment of control region sequences (300bp) without excluding sites with gaps. Ogilby (*Muntiacus reevesi*, GenBank accession number: AF527537) was used as out-group for phylogenetic tree construction. The NJ, MP and ML trees were performed using MEGA 5.05 (Tamura *et al.*, 2011). The MP tree was obtained using the Close-Neighbor-Interchange (CNI) with random sequence addition and with 1,000 bootstrap replicates. The most appropriate models of sequence evolution for ML trees were selected with MEGA. The best-fit model for ML tree was the Tamura 3-parameter model (T92) with Gamma distributed (+G) and proportion of Invariant sites (+I). The consensus ML trees were found by Nearest-Neighbor-Interchange (NNI) heuristic searches of 1,000 bootstrap replicates.

The median-joining network for was estimated using the

program Network version 4.6.1.2 (Bandelt *et al.*, 1999). Network analysis effectively describes the phylogenetic relationships among sequences, and allows inferring haplotype genealogies because they explicitly allow for extant ancestral sequences and alternative connections (Bandelt *et al.*, 1999).

Results

Genetic diversity and distance of Siberian musk deer

The 48 Siberian musk deer (35 sequences from NCBI and 13 from this study) presented 30 haplotypes based on 300bp of the hypervariable mtDNA control region. Estimates of genetic diversity in the studied populations are presented in Table 14. Four subspecies of Siberian musk deer did not share haplotypes from one another. Russian Far East subspecies (*M. m. turowi*) shared one common haplotypes (including 10 individuals) within population, but most of Siberia subspecies (*M. m. moschiferus*) showed unique haplotypes. Haplotype diversity (Hd) and nucleotide diversity (π) ranged from 0.62 and 0.4% in Russian Far East to 0.97 and 1.8% in Siberia. Relatively high level of nucleotide diversity (π= 1.3%) was detected in Korea subspecies (*M. m. parvipes*).

Calculation of the genetic distance and genetic differences between regions showed that the Korean subspecies as well as all other subspecies were closest to the Russian Far East (Table 15). Also, Korean and Siberia subspecies were relatively distant from Sakhalin Island (2.5~3%).

Table 14. Genetic diversity of Siberian musk deer from four different regions and subspecies.

Region	N	H	Hd	π(%)	Ref.
Korea *M. m. parvipes*	4	2	0.67	1.3	In this study
Russian Far East *M. m. turowi*	16	6	0.62	0.4	In this study (N= 8) Kholodova and Prikhodko (2006) Jang and Hwang (2010)
Siberia[a] *M. m. moschiferus*	23	19	0.97	1.8	In this study (N= 1) Kholodova and Prikhodko (2006)
Sakhalin Island *M. m. sachalinensi*	5	3	0.80	0.7	Kholodova and Prikhodko (2006)

N, number of sample; H, number of haplotype; Hd, Haplotype diversity;
π, nucleotide diversity; Ref, reference data; [a] Samples from Russia, Mongolia, china;

Table 15. Pairwise genetic distance (below diagonal) and pairwise genetic differences (above diagonal) between Siberian musk deer from different regions and subspecies.

	Korea	Russia Far East	Siberia	Sakhalin Island
Korea	–	4.394**	5.444**	7.000*
Russian Far East	0.015	–	4.663**	3.727*
Siberia[a]	0.019	0.016	–	8.514**
Sakhalin Island	0.025	0.013	0.030	–

[a] Samples from Russia, Mongolia, china;
* and **, respectively significant at 0.05 and 0.001 level.

Phylogenetic relationship of Siberian musk deer

Phylogenetic trees using NJ, MP and ML approaches generated similar tree topology. NJ tree was representatively presented in this study due to difficult to identify clade in the ML tree by short branch (Figure 15). Consistent with previous study (Kholodova and Prikhodko, 2006), Sakhalin Island (*M. m. sachalinensi*) was clearly identified and form a distinct cluster with high bootstrap support. Korean subspecies (*M. m. parvipes*) form a position between Siberian (*M. m. moschiferus*) and Russian Far East (*M. m. turowi*). These four subspecies did not share haplotypes and not mixed with each other in the phylogenetic tree. However, most of branches have low bootstrap support (under the 80%) except Sakhalin Island (*M. m. sachalinensi*).

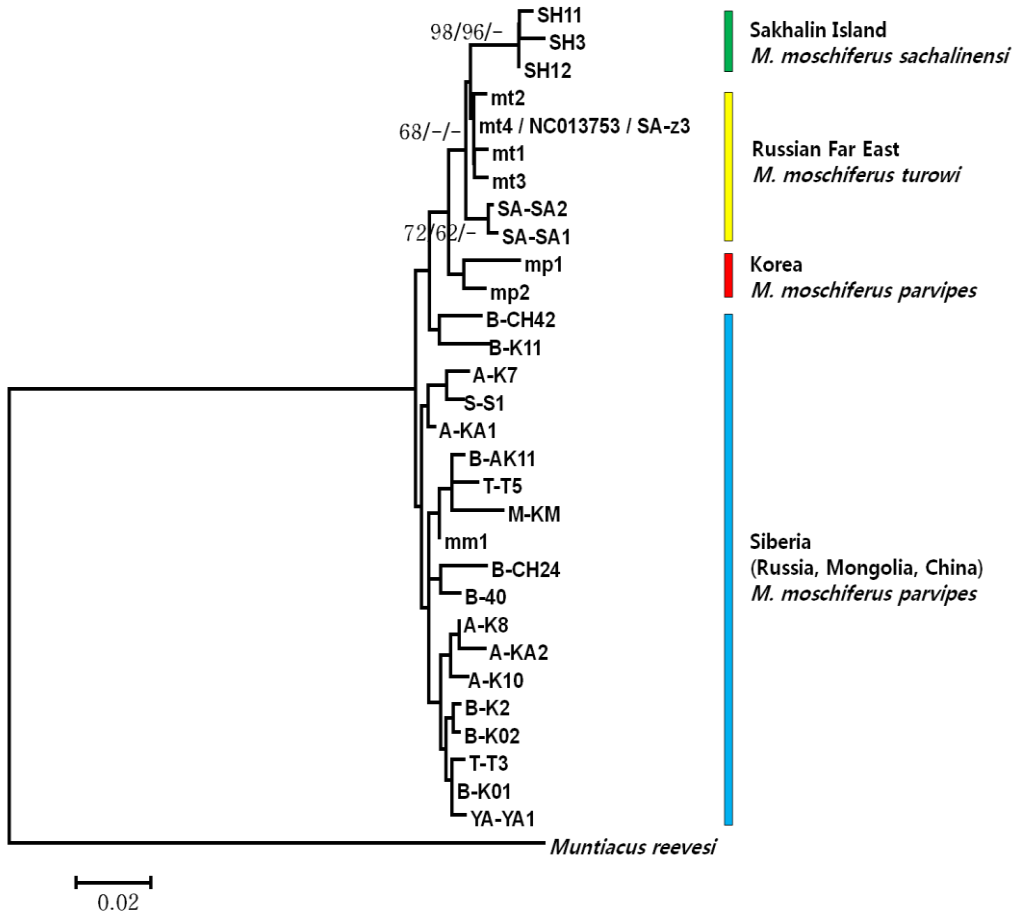


Figure 15. Phylogenetic tree of Siberian musk deer based on the mtDNA control region (300bp) of 30 haplotypes. Bootstrap values for NJ, MP and ML are shown for branches with over 60% support. See Figure 14 and Appendix S4 for location information.

Median-joining network analysis (Figure 16) of Siberian musk deer showed similar pattern with the previous study (Kholodova and Prikhodko, 2006). The Siberian subspecies (*M. m. moschiferus*) was connected with Russian Far East subspecies (*M. m. turowi*). Sakhalin Island (*M. m. sachalinensi*), Korean (*M. m. parvipes*) and Siberian subspecies (*M. m. moschiferus*) were not interconnected from each other, but were related to the Russian Far East. It was suggested that Korean and Sakhalin subspecies were originated from Russian Far East.

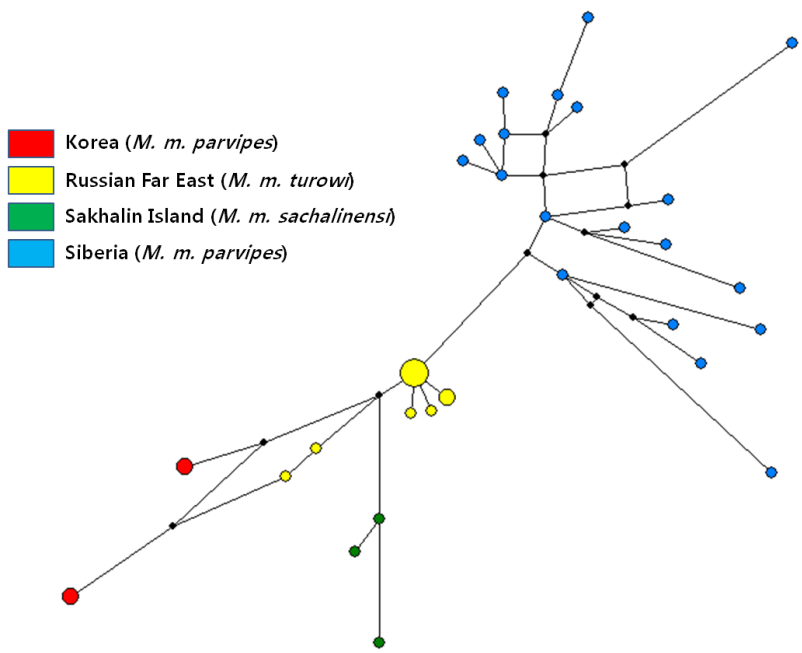


Figure 16. The median-joining network of Siberian musk deer based on the haplotype data of each individual. Branch lengths are scaled to the number of nucleotide substitutions and size of circles is proportional to the haplotype frequency (expect NCBI data). The black nodes denote inferred hypothetical haplotypes.

Discussion

In this study, we investigated and compared genetic parameters for subspecies from different parts of the regions of Siberian musk deer (*Moschus moschiferus*). Particularly, we focus on the genetic status of musk deer in South Korea and this is first report of Korean subspecies (*M. m. parvipes*) based on the molecular data.

Siberian musk deer (*M. moschiferus*) was divided three or five subspecies based on the morphological data (Groves *et al.*, 1995; Sokolov and Prikhod'ko, 1997, 1998; Groves and Grubb, 2011). According to different subspecies classification, musk deer inhabiting Korea were classified as a single subspecies or the same subspecies as that of Russian Far East and South Heilongjing. In the results of our study, Korean subspecies didn't share and mixed haplotypes with Russian Far East as well as other subspecies. However, bootstrap value of phylogenetic tree is very low except Sakhalin Island, and Korean subspecies are genetically closest to Russian Far East (Figure 15 and Table 15). Besides, it is possible that haplotype shared with Russian Far East cannot be found because of small number of samples of Korea, or it is possible that a part of mutually shared haplotype remains due to sharp population decline in Siberian musk deer. Therefore, it is unreasonable to discuss whether they belong to a single subspecies. Nevertheless, these results suggest that there is distinction pattern of haplotype composition among subspecies that supported by the phylogenetic trees and median-joining network.

What we should to focus on here is the origin of Siberian musk deer in Korea. Previous studies verified that musk deer of Russian Far East originated from Siberia which was an ancestral type, and musk deer in Sakhalin Island originated from Russian Far East (Kholodova and Prikhodko, 2006). In the network result, Korean (*M. m. parvipes*) was interconnected from other subspecies through the Russian Far East (*M. m. turowi*). It was possible to find that Korean subspecies also originated from Russian Far East form like Sakhalin Island subspecies (Figure 16). However, Korean subspecies formed a position between Russian Far East and Siberia subspecies in the phylogenetic tree with low bootstrap support (Figure 15). Therefore, this should be supported by studies through more samples that substantiate our results.

Musk deer in South Korea suffered dramatic decline of population and distribution during 6 decade (Lee and Rhim, 2002; Kim *et al.*, 2007; Park *et al.*, 2008). Nevertheless, they have maintained a significant amount of genetic diversity. When comparing the nucleotide diversity with similar sample size (Sakhalin Island), the Korean subspecies have relatively higher nucleotide diversity (1.3%) than Sakhalin Island (0.7%) (Table 14). The nucleotide diversity of Korean subspecies is comparable to the partial control region of Pampas deer, *Ozotoceros bezoarcticus* (1.1−2.5%) and Eld's deer, *Cervus eldi* (0−2.4%) which is considered endangered species as well (Gonzales *et al.*, 1998; Balakrishnan *et al.*, 2003). Moreover, this nucleotide diversity was also comparable to the abundance species, such as Siberian roe

deer, *Capreolus pygargus* (1.2–1.7%) and Sika deer, *Cervus nippon* (0.1–2.1%) (Lu *et al.*, 2006; Sheremetyeva *et al.*, 2010). High nucleotide diversity implies that this population probably had large effective population sizes in the recent past. However, if a decreased population size is maintained like Korean musk deer, it is obvious that genetic variability will be rapidly destroyed after more generations in the future (Nei *et al.*, 1975). Therefore, it is necessary to establish a suitable conservation plan for Siberian musk deer inhabiting Korea. But, this should be supported by study with more samples due to result of genetic diversity is accessible by the sample size.

The number of Korean musk deer is not accurately grasped. However, according to a previous report, the number is less than 30 individual and estimated that natural population restoration is difficult (Lee and Rhim, 2002; Kim *et al.*, 2007; Park *et al.*, 2008). Therefore, if restoration through artificial multiplication or restoration through re-introduction is needed, a population except musk deer in Korea should be considered. Thus we suggested musk deer of Russian Far-East (specifically Primorsky Krai), which are genetically close, as a potential population for restoring. The results of molecular phylogenetic study on other large mammal species in spite of different species show that there is no genetic difference from Russia Far East (Yunhaju) (Min *et al.*, 2004; Hong, 2005; Kim *et al.*, 2011). This can become an importance reference to musk deer restoration. Nevertheless our suggestion is only based on genetic relationship with short mitochondrial DNA (300bp).

Accordingly, it is necessary to prepare ecological basis through continuous studies on the food resources, habitat preference, natural environment and ecological features of musk deer inhabiting Russian Far East (specifically Primorsky Krai) and Korea (Crandall *et al.*, 2000).

General Discussion

In this study, genetic diversity, phylogeography and population genetic structure of Siberian roe deer (*Capreolus pygargus*) and genetic diversity, phylogeography of Siberian musk deer (*Moschus moschiferus*) were investigated using various molecular genetic markers.

The genetic diversity and phylogeography of Siberian roe deer, *Capreolus pygargus*, was demonstrated using mitochondrial DNA from Russia, Mongolia and South Korea. Most of Siberian roe deer populations had moderate level of haplotype and nucleotide diversity, except roe deer from Jeju Island, South Korea (SKJ) that showed the lowest level of genetic diversity. It is probable that a relatively small group of Siberian roe deer was founded in the island after the last glacial periods (founder effect), which led to reduced genetic diversity. Phylogenetic tree indicate that Siberian roe deer had four haplogroups, but they were not clearly described taxonomic ranges of subspecies and phylogeograpic distribution pattern. East Siberia regions (Mainland Korea, Russian Far East, Trans-Baikal region, Yakutia and Northern part of Mongolia) had various haplogroup and two haplogroups mainly exist in the west Siberia regions. Possible suggestion is that Trans-Baikal region (RSMG) and Amur region (RPRA) were geographical location of secondary colonization with high diversity, various haplogroups and demographic growth. Or else, mountains of the southern Siberia

were possible geographical range of putative ancestral group. In the previous study, Siberian roe deer from Jeju Island (SKJ) treat as *C. p. tianschanicus* (Koh *et al.*, 2000), or distinct subspecies (Koh and Randi, 2001; Park *et al.*, 2014). In this study, Jeju Island population (SKJ) was indeed genetically distinct from the all other regions, but they were not appeared to be distinct phylogenetic clade and distributed main haplogroup of western population. Therefore, Jeju Island population (SKJ) is in the process of being differentiated into subspecies, but has not yet been completely sorted phylogenetically. Therefore, it is diffecalt to discuss whether Jeju Island population (SKJ) belongs to distinct subspecies or not, and study of number of B-chromosome is necessary for distinct subspecies. Nevertheless Siberian roe deer from Jeju Island was composed of only one haplogroup and indicating homogeneous genetic composition due to the long term geographic isolation and small founder effect. Population of Siberian roe deer on Jeju Island is a unique one where conservation of one of the mitochondrial lineages.

The population genetic structure of Siberian roe deer was first trial using microsatellite marker. Our results reveal an apparent pattern of genetic differentiation among populations inhabiting Asia, showing moderate levels of genetic diversity compare to previously report for European roe deer. Siberian roe deer populations were significantly abated as a result of human interference during the 20th century. However, present data on the genetic diversity suggests that the historical population reduction was transient and insignificant. Microsatellite variability was consistently high in

populations from East and Central Asia compared to West Siberia. Reasonable factor is that areas to the south and east of Siberia had function as refugia for roe deer during glacial periods. Especially, roe deer from Jeju Island, South Korea (SKJ) showed the lowest level of genetic diversity among Siberian roe deer due to the geographic isolation, historical (founder effect) and recent (bottleneck) population fluctuations on Jeju Island. Therefore, continuous monitoring of genetic diversity would be essential for effective management and conservation of Siberian roe deer in Jeju Island. Genetic structure and differentiation among Siberian roe deer populations display the existence of genetically distinct three groups. These three groups were comprised of Southeastern group (Mainland Korea, Russian Far East, Trans-Baikal region and Northern part of Mongolia), Northwestern group (Western Siberia and Ural in Russia) and Jeju Island population. The genetic differentiation among groups separated primarily by major mountain ridges which played a role disturbing genetic flow of Siberian roe deer. These results suggest at least three distinct management units in the Siberian roe deer. On the other hand, genetic evidence also suggests an ongoing migration that may facilitate genetic admixture at the border areas between two groups. The insights obtained from this study shed light on management of Siberian roe deer in Asia and may be applied in conservation of local populations of Siberian roe deer.

The study of phylogeography for Siberian musk deer (*Moschus moschiferus*) in South Korea is first report of Korean subspecies

(*M. m. parvipes*) based on the molecular data. In this study, it was unreasonable to discuss whether Siberian musk deer in Korea belong to a single subspecies or not because of low bootstrap value in the phylogenetic tree and small sample size. Also Korean subspecies presented closest genetic distance and low genetic differentiation with Russian Far East (*M. m. turowi*). Nevertheless, results suggest that there was pattern of haplotype composition and haplotype distribution among subspecies. Also Siberian musk deer in Korea (*M. m. parvipes*) was interconnected from other subspecies through the Russian Far East (*M. m. turowi*) in the network that revealed Korean subspecies (*M. m. parvipes*) originated from Russian Far East, even though phylogenetic tree did not support the network. High nucleotide diversity and low haplotype diversity of Korean population compared with Sakhalin Island (small sample size) showed bottleneck in a formerly large, stable population (Grant and Bowen, 1998). Genetic variability will be rapidly reduced after more generations, if this small population size is maintained (Nei *et al.*, 1975). Therefore, it is necessary to establish a suitable conservation strategy for Siberian musk deer inhabiting Korea. Overall, musk deer of Russian Far East (specifically Primorsky Krai) is proper population for potential restoration, if restoration through artificial multiplication or re-introduction is needed.

In this study, investigate the genetic status of two ungulate species and discussed management and conservation of each species. When comparing phylogeography study of Siberian roe

deer and Siberian musk deer, equally two species from Korea were genetically closest with Russian Far East. However, Siberian roe deer was not clearly described taxonomic ranges of subspecies and phylogeograpic pattern, in contrast, Siberian musk deer had distinction pattern of haplotype composition among subspecies and Korea subspecies originated from Russian Far East in the network result. Also, Siberian roe deer and Siberian musk deer have totally different conservation status, but proper management and conservation of two species were suggested through genetic results in this study. Both species require effective population management such as constant population monitoring and population restoration. It is important to check the genetic status of population as well as carry out an ecological status of population for effective population management. Nevertheless our results are only based on genetic marker. Accordingly, it is necessary to prepare ecological basis through continuous studies on the natural environment and ecological features. Also, more samples with large or fine scale of genetic study will be necessary for supporting farther management and conservation plan.

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Appendices

Appendix S1. List of sample information of Siberian roe deer used for mitochondrial DNA and microsatellite analysis

ID	Location Code	Application	Country	Location	Year collected
644	SKJ	mtDNA	South Korea	Jeju Island	2004
647	SKJ	mtDNA	South Korea	Jeju Island	2004
650	SKJ	mtDNA	South Korea	Jeju Island	2004
1420	SKJ	mtDNA	South Korea	Jeju Island	2004
3422	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
3825	SKJ	mtDNA	South Korea	Jeju Island	2006
3943	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2001
4547	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2007
4981	SKJ	mtDNA	South Korea	Jeju Island	2007
4982	SKJ	mtDNA	South Korea	Jeju Island	2007
4983	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2007
5530	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2008
5888	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2008

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
5889	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2008
5890	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2008
8179	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8180	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8181	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8183	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8185	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8187	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8188	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8189	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8191	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8192	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8193	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8194	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
8195	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2006
9126	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2008
9127	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2008
9128	SKJ	Microsatellite	South Korea	Jeju Island	2009
9130	SKJ	Microsatellite	South Korea	Jeju Island	2009

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
9131	SKJ	Microsatellite	South Korea	Jeju Island	2009
9132	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2009
9133	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2009
9135	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2009
9136	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2009
9137	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2009
9138	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2009
9139	SKJ	mtDNA / microsatellite	South Korea	Jeju Island	2009
1838	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2004
2180	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2005
2216	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2005
2475	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2005
2679	SKM	mtDNA / microsatellite	South Korea	Chungcheongbuk-do	2005
3221	SKM	mtDNA / microsatellite	South Korea	Chungcheongnam-do	2006
3853	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
3890	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
3903	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
3927	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
4545	SKM	mtDNA / microsatellite	South Korea	Gyeongsangnam-do	2007

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
4546	SKM	mtDNA	South Korea	Gangwondo	2007
4653	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007
4654	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007
4824	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007
4825	SKM	mtDNA	South Korea	Gangwondo	2007
4826	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007
4827	SKM	mtDNA	South Korea	Gangwondo	2007
4912	SKM	mtDNA	South Korea	Gyeonggi-do	2007
8197	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
8198	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
8199	SKM	Microsatellite	South Korea	Gangwondo	2006
8200	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007
8201	SKM	Microsatellite	South Korea	Gangwondo	2005
8202	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007
8203	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2005
8204	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2005
8205	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007
8206	SKM	Microsatellite	South Korea	Gangwondo	2007
8207	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2007

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
8208	SKM	Microsatellite	South Korea	Gangwondo	2007
8211	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
8212	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2006
9124	SKM	mtDNA / microsatellite	South Korea	Gangwondo	2009
9125	SKM	Microsatellite	South Korea	Gangwondo	2009
2145	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray	2003
3792	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray , Lozo, Kiyevka river	2006
3793	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray , Lozo, Kiyevka river	2006
3794	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray , Lozo, Kiyevka river	2006
4227	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray , Lozo, Kiyevka river	2007
4228	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray , Lozo, Kiyevka river	2007
4229	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray , Lozo, Kiyevka river	2007
4230	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray , Lozo, Kiyevka river	2007
4779	RPR	mtDNA	Russia	River Bikin, north Primorie	2007
4780	RPR	mtDNA	Russia	River Bikin, north Primorie	2007
4781	RPR	mtDNA	Russia	River Bikin, north Primorie	2007
4782	RPR	mtDNA	Russia	River Bikin, north Primorie	2007
4784	RPR	mtDNA	Russia	River Bikin, north Primorie	2007
4785	RPR	mtDNA / microsatellite	Russia	River Bikin, north Primorie	2007

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
4786	RPR	mtDNA / microsatellite	Russia	River Bikin, north Primorie	2007
4788	RPR	mtDNA / microsatellite	Russia	Chuguyevka, south Primorie	2007
4789	RPR	mtDNA / microsatellite	Russia	Chuguyevka, south Primorie	2007
4790	RPR	mtDNA / microsatellite	Russia	Chuguyevka, south Primorie	2007
4792	RPR	mtDNA	Russia	Chuguyevka, south Primorie	2007
4793	RPR	mtDNA	Russia	Chuguyevka, south Primorie	2007
4794	RPR	mtDNA	Russia	Chuguyevka, south Primorie	2007
4795	RPR	mtDNA	Russia	Chuguyevka, south Primorie	2007
4796	RPR	mtDNA	Russia	Primorskiy Kray, Lozo, Kiyevka river	2007
4797	RPR	mtDNA	Russia	Primorskiy Kray, Lozo, Kiyevka river	2007
4798	RPR	mtDNA	Russia	Primorskiy Kray, Lozo, Kiyevka river	2007
4799	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray, Lozo, Kiyevka river	2007
4801	RPR	mtDNA	Russia	Primorskiy Kray, Lozo, Kiyevka river	2007
5202	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2008
5203	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2008
5204	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2008
5205	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Sokolovka River	2008
5206	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2008
5207	RPR	mtDNA / microsatellite	Russia	Primorskiy Kray, Lozo, Kiyevka river	2008

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
9831	RPR	Microsatellite	Russia	Primorsky Krai, Lazo, Sokolovka River	2009
9832	RPR	Microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2009
9833	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2009
9834	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2009
9835	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2009
9836	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Lazo, Lazovka River	2009
9837	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Terney, Tajozhnaja river	2009
9838	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Terney, Tajozhnaja river	2009
9839	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Terney, Tajozhnaja river	2009
9840	RPR	mtDNA / microsatellite	Russia	Primorsky Krai, Terney, Serebrianka River	2009
4769	RAM	mtDNA	Russia	River Nora, Amur region	2006
4770	RAM	mtDNA	Russia	River Nora, Amur region	2006
4771	RAM	mtDNA	Russia	River Nora, Amur region	2006
4772	RAM	mtDNA	Russia	River Nora, Amur region	2006
4773	RAM	mtDNA	Russia	River Nora, Amur region	2006
4774	RAM	mtDNA	Russia	River Nora, Amur region	2006
4775	RAM	mtDNA	Russia	River Nora, Amur region	2006
4776	RAM	mtDNA	Russia	River Nora, Amur region	2006
4777	RAM	mtDNA	Russia	River Nora, Amur region	2006

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
4778	RAM	mtDNA	Russia	River Nora, Amur region	2006
11175	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2010
11176	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2010
11177	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2010
11178	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2010
11270	RYA	mtDNA / microsatellite	Russia	Hangalasskii region, Yakutia	2009
11271	RYA	mtDNA / microsatellite	Russia	Hangalasskii region, Yakutia	2009
11272	RYA	mtDNA / microsatellite	Russia	Hangalasskii region, Yakutia	2009
11273	RYA	mtDNA / microsatellite	Russia	Hangalasskii region, Yakutia	2009
11274	RYA	mtDNA / microsatellite	Russia	Hangalasskii region, Yakutia	2009
11275	RYA	mtDNA / microsatellite	Russia	Mountain region, Yakutia	2009
13459	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2011
13460	RYA	microsatellite	Russia	Central Yakutia	2011
13461	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2011
13462	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2011
13463	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2011
13464	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2011
13465	RYA	microsatellite	Russia	Central Yakutia	2011
13466	RYA	mtDNA	Russia	Central Yakutia	2011

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
13467	RYA	mtDNA / microsatellite	Russia	Central Yakutia	2011
13468	RYA	mtDNA	Russia	Central Yakutia	2011
13469	RYA	mtDNA	Russia	Central Yakutia	2011
13470	RYA	mtDNA	Russia	Central Yakutia	2011
13471	RYA	mtDNA	Russia	Central Yakutia	2011
13472	RYA	mtDNA	Russia	Central Yakutia	2011
13473	RYA	mtDNA	Russia	Central Yakutia	2011
13475	RYA	mtDNA	Russia	Central Yakutia	2011
4619	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4620	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4621	RSO	mtDNA	Russia	Sokhondinsky	2003
4622	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4623	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4624	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4625	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4626	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4627	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
4628	RSO	mtDNA / microsatellite	Russia	Sokhondinsky	2003
11265	RAL	microsatellite	Russia	Russia, mount.Altay	2009

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
11266	RAL	microsatellite	Russia	Russia, mount.Altay	2009
11267	RAL	mtDNA / microsatellite	Russia	Russia, mount.Altay	2009
11268	RAL	mtDNA / microsatellite	Russia	Russia, mount.Altay	2009
11269	RAL	mtDNA / microsatellite	Russia	Russia, mount.Altay	2009
11276	RNO	mtDNA / microsatellite	Russia	Novosibirsk region	2009
11277	RNO	mtDNA / microsatellite	Russia	Novosibirsk region	2009
11278	RNO	mtDNA / microsatellite	Russia	Novosibirsk region	2009
11279	RNO	mtDNA / microsatellite	Russia	Novosibirsk region	2009
11280	RNO	mtDNA / microsatellite	Russia	Novosibirsk region	2009
11281	RNO	mtDNA / microsatellite	Russia	Novosibirsk region	2009
11282	RNO	microsatellite	Russia	Novosibirsk region	2009
5976	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
5977	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
5978	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
5979	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
5980	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
5981	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
5982	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
5983	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008

Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
5984	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2008
11140	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2007
11149	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11150	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11151	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11152	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11153	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11154	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11155	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11156	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11157	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11158	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11159	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11160	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2009
11300	RUR	mtDNA / microsatellite	Russia	Ural Sverdlovskaya oblast	2010
11141	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007
11142	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007
11143	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007
11144	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007

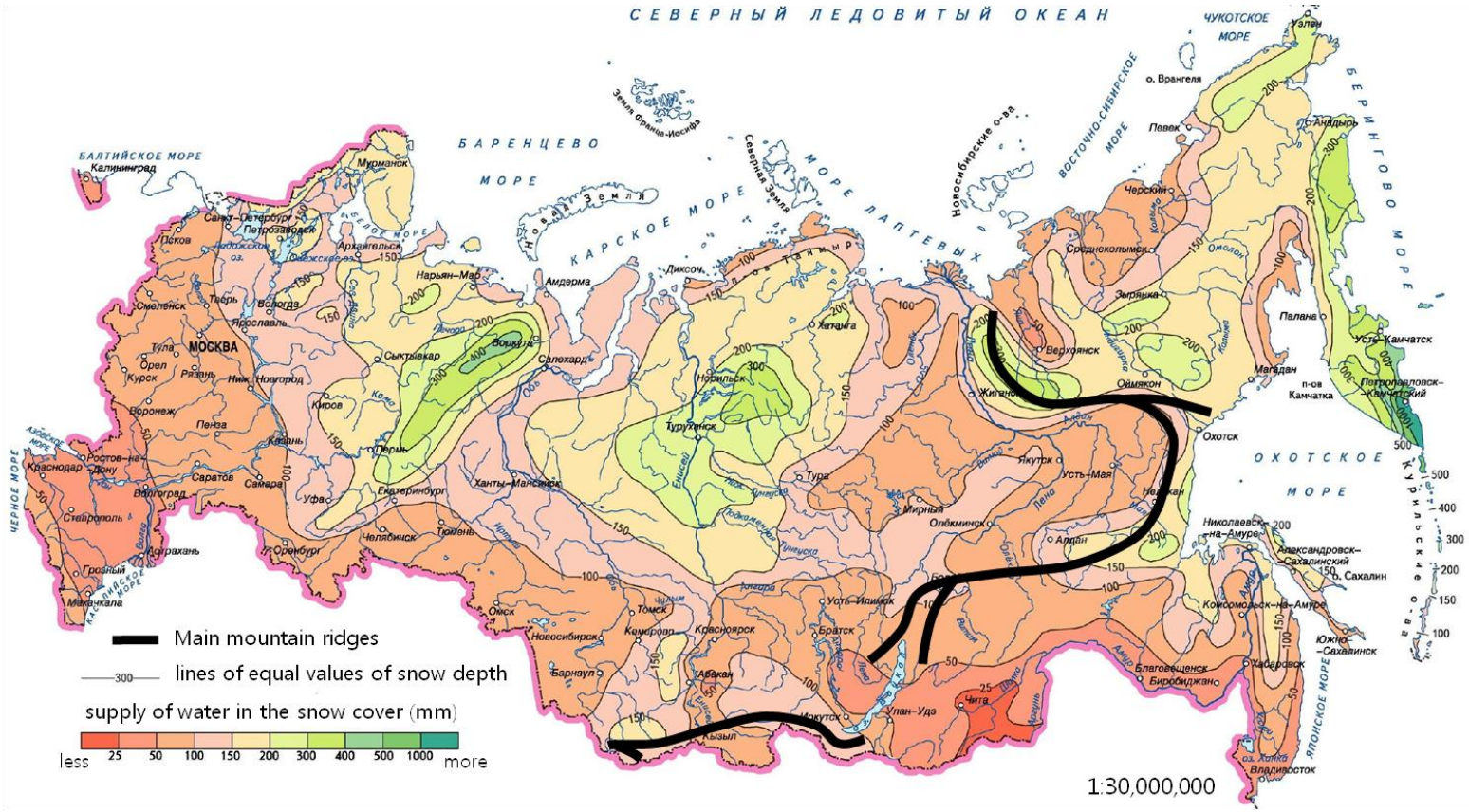
Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
11145	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007
11146	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007
11147	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007
11148	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2007
11161	RKU	microsatellite	Russia	Kurganskaya	2009
11162	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11163	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11164	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11165	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11166	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11167	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11168	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11169	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11170	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11171	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11172	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
11173	RKU	mtDNA / microsatellite	Russia	Kurganskaya	2009
9841	ROR	mtDNA	Russia	Orenburg, Belyaevski district	2006
9842	ROR	mtDNA	Russia	Orenburg, Belyaevski district	2006

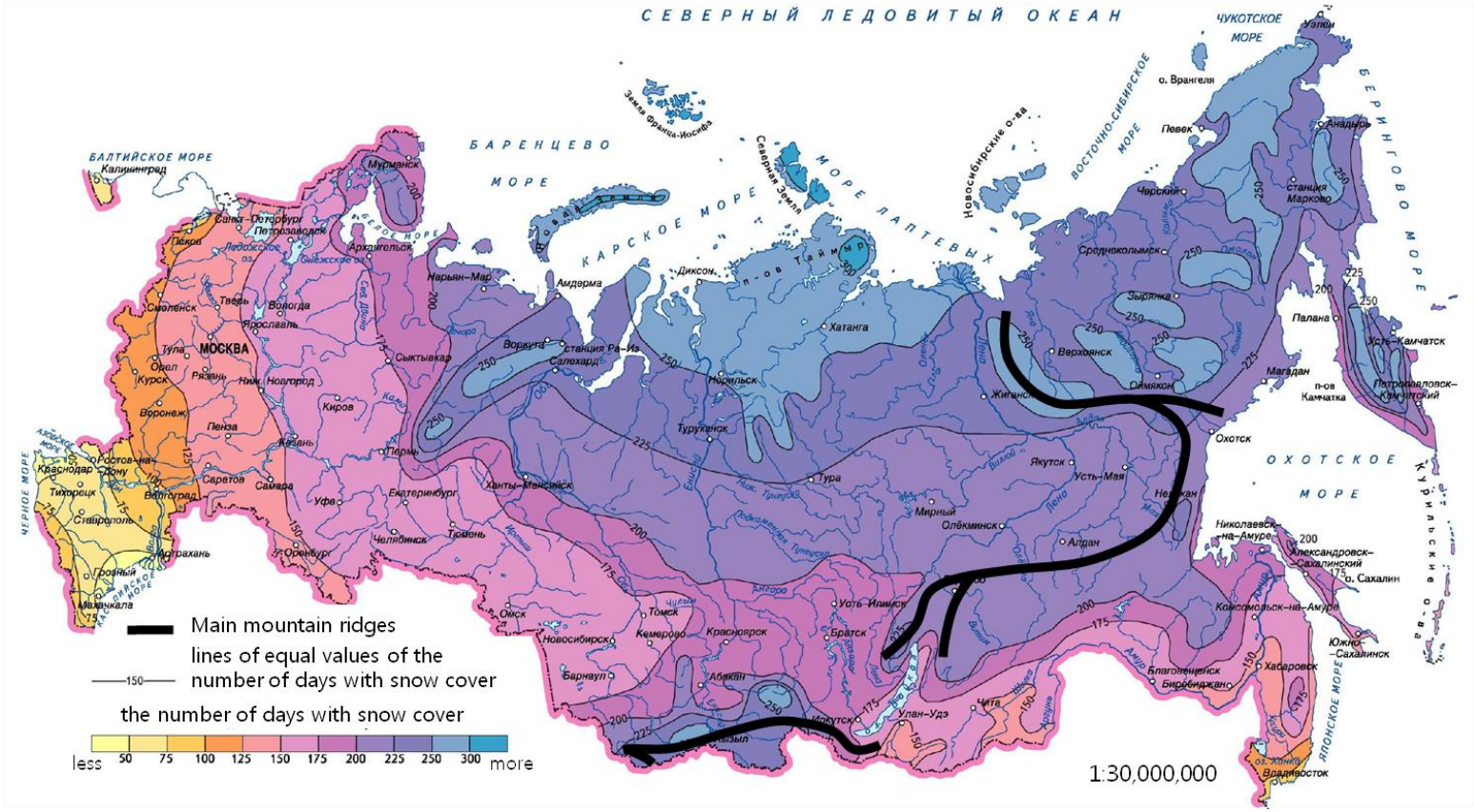
Appendix S1. (Continued)

ID	Location Code	Application	Country	Location	Year collected
9843	ROR	mtDNA	Russia	Orenburg, Belyaevski district	2006
3161	MGN	mtDNA / microsatellite	Mongolia	–	2006
5917	MGN	mtDNA / microsatellite	Mongolia	–	2008
11664	MGN	mtDNA / microsatellite	Mongolia	–	2010
11666	MGN	mtDNA / microsatellite	Mongolia	–	2010
11667	MGN	mtDNA / microsatellite	Mongolia	–	2010
11668	MGN	mtDNA / microsatellite	Mongolia	–	2010
11669	MGN	mtDNA / microsatellite	Mongolia	–	2010
11670	MGN	mtDNA / microsatellite	Mongolia	–	2010
11671	MGN	mtDNA / microsatellite	Mongolia	–	2010
11672	MGN	mtDNA / microsatellite	Mongolia	–	2010
11673	MGN	mtDNA / microsatellite	Mongolia	–	2010
12542	MGN	mtDNA / microsatellite	Mongolia	–	2010

Appendix S2. Value of snow depth (supply of water in the snow cover) and main mountain ridge in the Russia. Map and data are sourced from Russian website (http://geographyofrussia.com)



Appendix S3. The number of days with snow cover and main mountain ridge in the Russia. Map and data are sourced from Russian website (http://geographyofrussia.com)



Appendix S4. List of sample information of Siberian musk deer used for mitochondrial DNA analysis. Published 35 sequences were obtained from GenBank.

Site No.	ID	Haplotypes	Country	Locality	Accession No.	Reference
1	15255	mp1	Korea	Gangwon-do, Hwacheon-gun		This study
1	15257	mp1	Korea	Gangwon-do, Hwacheon-gun		This study
1	15258	mp2	Korea	Gangwon-do, Hwacheon-gun		This study
1	15373	mp2	Korea	Gangwon-do, Hwacheon-gun		This study
2	501	mt1	Russia	Primorsky Krai		This study
2	521	mt4	Russia	Primorsky Krai, Terney district, serebrianka		This study
2	5209	mt2	Russia	Primorsky Krai, Lazo district, Lazovka River		This study
2	5210	mt3	Russia	Primorsky Krai, Lazo district, Lazovka River		This study
2	9809	mt4	Russia	Primorsky Krai, Lazo district, Celinka River		This study
2	9810	mt4	Russia	Primorsky Krai, Lazo district, Celinka River		This study
2	9811	mt4	Russia	Primorsky Krai, Terney district, Zabolochennaja		This study
2	11036	mt1	Russia	Primorsky Krai, Lazo district, Celinka River		This study
2	PRI01	NC013753	Russia	Primorsky Krai	NC_013753	Jang and Hwang (2010)
3	79	mm1	China	Heilong jiang		This study
4	ALT01	A-KA1	Russia	Altai, Pyzha River bassin	DQ269165	Kholodova and Prikhodko (2006)

Appendix S4. (Continued)

Site No.	ID	Haplotypes	Country	Locality	Accession No.	Reference
4	ALT02	A-KA2	Russia	Altai, Pyzha River bassin	DQ269166	Kholodova and Prikhodko (2006)
4	ALT03	A-K7	Russia	Altai, Pyzha River bassin	DQ269168	Kholodova and Prikhodko (2006)
4	ALT04	A-K8	Russia	Altai, head of the Kachesh River	DQ269167	Kholodova and Prikhodko (2006)
4	ALT05	A-K10	Russia	Altai, Bashelaksk Mountain Range	DQ269171	Kholodova and Prikhodko (2006)
5	WSA01	S-S1	Russia	Western Sayan	DQ269169	Kholodova and Prikhodko (2006)
6	SAY01	T-T3	Russia	Tyva, Western Sayan	DQ269181	Kholodova and Prikhodko (2006)
6	SAY02	T-T5	Russia	Tyva, Western Sayan	DQ269182	Kholodova and Prikhodko (2006)
7	IRK01	B-K01	Russia	Irkutsk oblast, Khargino Mountain Range	DQ269173	Kholodova and Prikhodko (2006)
7	IRK02	B-K02	Russia	Irkutsk oblast, Khargino Mountain Range	DQ269174	Kholodova and Prikhodko (2006)
7	IRK03	B-K2	Russia	Irkutsk oblast, Khargino Mountain Range	DQ269172	Kholodova and Prikhodko (2006)
7	IRK04	B-K2	Russia	Irkutsk oblast, Khargino Mountain Range	DQ269172	Kholodova and Prikhodko (2006)
7	IRK05	B-K01	Russia	Irkutsk oblast, Khargino Mountain Range	DQ269173	Kholodova and Prikhodko (2006)
7	IRK06	B-K2	Russia	Irkutsk oblast, Khargino Mountain Range	DQ269172	Kholodova and Prikhodko (2006)

Appendix S4. (Continued)

Site No.	ID	Haplotypes	Country	Locality	Accession No.	Reference
7	IRK07	B-K2	Russia	Irkutsk oblast	DQ269172	Kholodova and Prikhodko (2006)
7	IRK08	B-K11	Russia	Irkutsk oblast, outskirts of the village of Nekrasovka	DQ269179	Kholodova and Prikhodko (2006)
8	BUR01	B-AK11	Russia	Buryatia	DQ269170	Kholodova and Prikhodko (2006)
8	BUR02	B-CH24	Russia	Buryatia, Zakamensk raion	DQ269175	Kholodova and Prikhodko (2006)
8	BUR03	B-CH42	Russia	Buryatia, Zakamensk raion	DQ269176	Kholodova and Prikhodko (2006)
9	MON01	M-KM	Mongolia	Mongolia	DQ269178	Kholodova and Prikhodko (2006)
10	CHI01	B-40	Russia	Chita Oblast, head of the Kyra River	DQ269177	Kholodova and Prikhodko (2006)
11	YAK01	YA-YA1	Russia	Republic of Sakha (Yakutia), Ust-Aldan raion, outskirts of the settlement of Kepteni	DQ269180	Kholodova and Prikhodko (2006)
12	SIK01	SA-SA1	Russia	Sikhote-Alin', Primorskii krai	DQ269185	Kholodova and Prikhodko (2006)
12	SIK02	SA-SA2	Russia	Sikhote-Alin'	DQ269183	Kholodova and Prikhodko (2006)
12	SIK03	SA-z3	Russia	Sikhote-Alin', Taezhka	DQ269184	Kholodova and Prikhodko (2006)
12	SIK04	SA-z3	Russia	Sikhote-Alin', Taezhka	DQ269184	Kholodova and Prikhodko (2006)
12	SIK05	SA-z3	Russia	Sikhote-Alin', Taezhka	DQ269184	Kholodova and Prikhodko (2006)

Appendix S4. (Continued)

Site No.	ID	Haplotypes	Country	Locality	Accession No.	Reference
12	SIK06	SA−z3	Russia	Sikhote−Alin', Wide fold	DQ269184	Kholodova and Prikhodko (2006)
12	SIK07	SA−z3	Russia	Sikhote−Alin', Wide fold	DQ269184	Kholodova and Prikhodko (2006)
13	SAK01	SH3	Russia	Sakhalin Island, Nabil skii Mountain Range, head of the Gromova River	DQ269187	Kholodova and Prikhodko (2006)
13	SAK02	SH3	Russia	Sakhalin Island, Makarov raion, head of the Makarov River	DQ269187	Kholodova and Prikhodko (2006)
13	SAK03	SH11	Russia	Sakhalin Island, Western Sakhalin Mountains, Aleksandrovsk−Sakhalin raion	DQ269186	Kholodova and Prikhodko (2006)
13	SAK04	SH12	Russia	Sakhalin Island, Tymovsk raion, head of the Tym' River, Eastern Sakhalin Mountains	DQ269188	Kholodova and Prikhodko (2006)
13	SAK05	SH12	Russia	Sakhalin Island, Tymovsk raion, head of the Tym' River, Eastern Sakhalin Mountains	DQ269188	Kholodova and Prikhodko (2006)

시베리아노루의 계통지리 및 집단유전학 연구와 시베리아사향노루의 계통지리연구

서울대학교 대학원

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국문초록

노루 (노루속; *Capreolus*)는 구북구에 가장 널리 퍼져있는 중형 포유동물 중 하나이며, 주로 유럽에 서식하는 유럽노루 (*C. capreolus*)와 아시아 대륙에 서식하는 시베리아노루 (*C. pygargus*), 이렇게 2종을 포함한다. 유럽노루에 대한 유전적 연구는 많이 되어 있지만 시베리아노루의 유전적 다양성과 유전적 유연관계에 대한 연구는 미비하며 microsatellite 좌위 분석을 통하여 진행된 적은 없다. 따라서 본 연구에서는 미토콘드리아 DNA와 microsatellite 마커를 이용하여

시베리아노루의 계통지리와 유전적 다양성, 유전적 집단구조를 조사하였다. 먼저 계통지리와 유전적 다양성의 연구에서는 러시아, 몽골, 한국 등의 12개 지역 (7그룹)에서 채집한 219개체의 미토콘드리아 조절부위 (control region) (963bp)와 사이토크롬*b* (1,140bp)를 합친 염기서열을 사용했다. 미토콘드리아 조절부위를 다른 사슴과 (Family Cervidae) 종에서 보고된 다양성과 비교하였을 때, 대부분의 시베리아노루 집단에서 보통 수준의 haplotype 다양성 (Hd)과 뉴클레오타이드 다양성 (π)을 나타냈다. 하지만 제주도의 시베리아노루집단은 가장 낮은 다양성을 보였으며 다른 지역의 시베리아노루와도 먼 유전적 거리를 보였다. 이는 제주도 노루집단이 장기간 지리적으로 격리되었고 집단감소나 창시자효과 때문으로 여겨진다. 비록 제주도의 시베리아노루 집단이 계통도에서 별개의 분기군을 나타내지 않았지만, 하나의 미토콘드리아 계통이 존재하는 유일한 곳이며 더 이상의 유전자 흐름은 기대하기 힘들기 때문에 보전이 필요하다. 계통도와 네트워크 분석에서 나머지 지역의 시베리아노루는 유전적으로 아종의 분류범위나 계통지리적 분포 패턴이 나타내지 않았다. 하지만 4개의 haplogroup을 가지고 있으며, 동부 시베리아 지역은 다양한 haplogroup이 분포하였고 서부 시베리아 지역은 2개의 haplogroup이 주로 분포하였다. 바이칼 지역과 아무르 지역은 모든 haplogroup의 분포, 집단 팽창의 존재, 높은 유전적 다양성을 보였다. 따라서 남부 시베리아의 산맥 부근에 조상그룹이 존재했을 가능성이 있거나 어딘가에 존재했던 조상그룹의 분산 후 바이칼 지역과 아무르 지역에 두 번째 군집을 형성했을 것으로 추정된다.

시베리아노루의 집단의 유전적 구조와 유전적 변이 정도를 조사하기 위해, 12 microsatellite 좌위를 아시아전역에서 채집한 189샘플을 대상으로 분석하였다. 결과는 가장 낮은 유전적 다양성 (Ar= 2.2, H_E=

0.39)을 나타낸 제주집단을 제외하고 거의 대부분의 집단에서 보통 수준의 유전적 다양성 (Ar= 2.8–3.7, H_E= 0.52–0.63)을 나타내었다. 서부지역의 집단들 (평균 Ar= 2.9, H_E= 0.54)을 동부지역의 집단 (평균 Ar= 3.5, H_E= 0.60)과 비교해보았을 때 비교적 낮은 유전적 다양성을 보였으며, 높은 수준의 유전적 분화 (평균 pairwise F_{ST}= 0.122)를 나타내었다. 또한 시베리아노루는 유전적으로 뚜렷이 구분되는 3개의 그룹이 존재했다. 이 3 그룹은 남동부 그룹 (한국본토, 러시아 극동지방, 바이칼 지역, 몽골북부), 북서부 그룹 (시베리아 서부와 러시아 우랄) 그리고 제주도 집단으로 구성되어 있었다. 섬으로 분리된 제주도를 제외한 나머지 두 그룹의 유전적 분화는 산맥 (알타이, 사이얀, 스타노보이, 콜리마 산맥)을 사이에 두고 나타났고, 다른 여러 분석들 (Barrier, AMOVA, F_{ST}, gene flow) 도 산맥에 의한 유전적 분화를 지지하는 결과를 나타내었다. 한편, 이 두 그룹의 경계 지역에서는 두 유전자 타입이 혼재되어 나타났는데 이는 경계지역에서 그룹간 이주가 일부 있다는 증거로 생각된다. 종합적으로, 비록 경계지역에 유전자 혼재가 존재하지만 시베리아노루 집단은 유전적으로 구분되는 3개의 그룹이 존재하고 이들의 관리와 보전을 위해서 아시아 지역에 적어도 3개의 보전단위 설정을 제안하는 바이다.

시베리아사향노루 (*Moschus moschiferus*)는 국제적으로 멸종위기에 처한 종이다. 멸종위기에 처한 가장 큰 이유는 인간에 의한 남획과 서식지 감소이다. 이들은 Moschidae과 *Moschus*속에서 가장 넓게 분포하는 종 중 하나다. 과거 한국에는 사향노루집단이 태백산을 따라서 분포하였으며 산맥을 따라 비교적 큰 집단이 서식하고 있었을 것으로 추정한다. 하지만 한국에 서식하는 시베리아사향노루 집단(*M. m. parvipes*)의 분포지역은 1950년대부터 1999년까지 급격히 감소하였고, 따라서 효과적인 보전이 필요하다. 성공적이고 올바른 종 보전을 위해,

집단의 유전적 특징 및 유전적 다양성 정도를 확인하는 것이 중요하다. 유전자 분석은 유전적 다양성, 유전적 유연관계 등의 정보를 제공할 수 있으며 알맞은 복원과 보전프로그램을 뒷받침해 줄 수 있다. 따라서 한국에 서식하는 시베리아사향노루 아종과 다른 아종과의 유전적 유연관계를 조사하기 위해, 3 지역과 아종 (극동 러시아, *M. m. turovi*; 중국 북동부, *M. m. moschiferus*; 한국, *M. m. parvipes*)에서 채집한 13개 털과 DNA 샘플에서 미토콘드리아 조절부위 (control region) (300bp)를 추출하였다. 또한 아종, 지역집단 사이에 포괄적인 유전적 유연관계를 확인하기 위해 이미 출판된 논문의 35개 미토콘드리아 조절부위 염기서열을 (300bp) NCBI에서 얻어 함께 분석에 사용하였다. 본 연구 결과, 한국의 사향노루는 Russian Far East와 가장 가까운 유전적 거리 (0.015)를 보였다. 계통도에서 각 아종은 (한국, 극동러시아, 시베리아, 사할린 사향노루) 서로 haplotype을 공유하지 않았고 haplotype의 분포가 섞이지 않았다. haplotype 구성에 지역간, 아종간 경향성을 나타내었다. 하지만 사할린 사향노루를 제외하고 모든 계통도 가지가 낮은 bootstrap 지지도를 나타내었고 개체수도 적기 때문에 아종 단위 구분을 논하기에는 무리가 있었다. 네트워크 결과는 한국의 시베리아 사향노루 집단이 러시아 극동지방에서 기원 되었다고 나타났다. 한국의 시베리아사향노루 (4개체)를 샘플 수가 비슷한 사하린 사향노루 (5개체)와 비교하였을 때 높은 뉴클레오타이드 다양성 (π= 1.3%)과 낮은 haplotype다양성 (Hd= 0.67)을 보였다. 이런 유전자 다양성 패턴은 크고 안정된 집단이 급격한 병목현상 (집단감소)을 겪었을 때 나타나며 한국의 시베리아사향노루 아종이 이와 같은 현상을 겪었을 것이라고 생각된다. 만약 감소된 집단 크기가 계속 유지된다면, 좀 더 여러 세대가 지난 뒤에 급격한 유전적 다양성의 감소가 일어날 수 있다. 따라서 한국 시베리아사향노루의 보전 및 관리가 필요하며 국내에

서식하는 사향노루를 이용한 개체수 복원 및 증식이 가장 좋은 방법일 것이다. 하지만 이것이 불가능 하다면, 한국 집단의 기원이며 유전적으로 가까운 러시아 극동지역 (특히 러시아 프리모스키 지역)의 시베리아사향노루 집단을 한국 사향노루의 복원을 위한 잠정적 집단으로 제안할 수 있다. 그러나 성공적인 보전을 위해서는 추후 더 많은 샘플을 통한 유전학적 연구와 다양한 생태학적 연구가 필요하다고 생각한다.

본 연구는 우제목에 속하는 시베리아노루와 시베리아사향노루의 유전적 분석을 통해 유전자 다양성, 계통지리와 집단구조를 규명하였다. 두 종의 미토콘드리아유전자를 통한 계통지리 연구결과를 비교해보면, 한국의 시베리아사향노루와 시베리아노루는 공통적으로 극동러시아와 유전적으로 가장 가까웠다. 다른점으로는, 시베리아노루는 아종이나 지리적으로 분화된 계통을 나타내지 않아 계통지리적 패턴을 알 수 없었던 반면, 시베리아사향노루는 haplotype의 구성에서 아종간 경향을 나타냈으며 네트워크 분석결과에서는 한국의 시베리아사향노루가 극동러시아를 통해 기원했을 가능성을 보여주었다. 또한 본 연구에서는 시베리아노루와 시베리아사향노루의 보전 및 관리를 뒷받침해 줄 수 있는 유전학적 연구 결과를 제시하였다.

주요어: 시베리아노루, 시베리아사향노루, 유전적 다양성, 계통지리학, 멸종위기종, 미토콘드리아DNA, Microsatellite마커, 관리단위

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